

**CHARACTERISATION AND MONITORING OF
PLASMID DNA VECTORS OF INCREASING SIZE
DURING PROCESSING FOR GENE THERAPY AND DNA
VACCINATION APPLICATIONS**

by

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ABSTRACT

The cell lysis step is one of the most critical operations in large-scale plasmid DNA processing as fragments of chromosomal DNA and degraded plasmids remaining in solution have a detrimental effect on downstream operations. In the present thesis the effect of the engineering environment on the bacterial cell suspension used in the lysis step and on solutions containing gene vectors of up to 242 kb in size was investigated. An integral aspect of the thesis was to evaluate current analytic techniques relating to the characterisation of process streams containing plasmid vectors and to identify and develop characterisation techniques suitable for process development and manufacturing.

Cell pastes derived from laboratory- and 450 litre- scale fermentations of plasmid-containing bacterial cells were characterised using a variety of analytical techniques. The impact of pilot scale continuous centrifugation equipment used for cell harvesting was then investigated. It was observed that equipment design and operating conditions had an impact on product yield and the molecular weight of contaminant DNA in the process stream. It was found that direct measurement of released nucleic acids at the cell resuspension stage can provide a simple analytical technology to characterise cells before the lysis stage.

After cell lysis, the downstream options depend on the susceptibility of the product to chemical and mechanical damage in solution. In the case of circular DNA molecules, it is of particular importance to characterise the backbone integrity. A microwell-based high throughput method to evaluate this for pure double stranded DNA solutions was developed. The method was then validated for reproducibility, accuracy and sensitivity and was successfully adapted for use on a robotic liquid handling system. The method was utilised to investigate the response to hydrodynamic stresses of bacterial artificial chromosomes. It was observed that for a 116 kb vector, average shear rates $\geq 10^4 \text{ s}^{-1}$ (comparable with standard process equipment), caused a 5 fold decrease in the integrity of the molecules in the sample.

Finally, the thesis ends with a look towards future process analytical technologies and assay development within the context of DNA-based pharmaceuticals.

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LIST OF ABBREVIATIONS USED IN THE THESIS

AAV – Adeno associated virus

AEC- Anion Exchange High performance Liquid Chromatography

AGE – Agarose Gel Electrophoresis

AIDS - Acquired Immune Deficiency Syndrome

AV – Adenovirus

A_{Xnm} – Absorbance, at X nanometers

BAC - Bacterial artificial chromosome

CBER – Center for Biological Evaluation and Research

CCC - Covalently closed circle DNA

CDER – Center for Drug Evaluation and Research

cDNA - Chromosomal DNA

CE – Capillary Electrophoresis

CEC – Capillary Electro-chromatography

CGE – Capillary Gel Electrophoresis

CV – coefficient of variation (calculated as mean / standard deviation)

DNA - Deoxyribonucleic acid

dsDNA – Double stranded DNA

E. coli – Escherichia coli

EFPIA - European Federation of Pharmaceutical Industries and Associations

ELISA – Enzyme Linked Immunosorbent Assay

F - Fluorescence

FDA – United States Food and Drug Administration

GFC – Gel Filtration Chromatography

h - hour

HIV - Human Immunodeficiency Virus

HPCE – High performance Capillary Electrophoresis

HPLC – High performance Liquid Chromatography

IND – Investigational New Drug

IPA – Isopropanol precipitated

LAL – Limulus amoebocyte lysate

LPS – Lipopolysaccharide

LV – Lentivirus

min - minute

OC, ocDNA – Opencircular DNA

OD_{xnm} – Optical Density, at X nanometers

PAT – Process Analytical Techniques

pDNA – Plasmid DNA

PESDA – Perfluorocarbon exposed sonicated dextrose albumin

PFGE – Pulsed Field Gel Electrophoresis

PLGA – Poly lactic- co – glycolic acid

qPCR – Quantitative Polymerase Chain Reaction

RF – Relative Fluorescence

RNA – Ribonucleic acid

RO – Reverse Osmosis

RV – Retrovirus

SC, scDNA – Supercoiled DNA

SDS – Sodium Dodecyl Sulphate

SEC – Size exclusion chromatography

ssDNA – Single stranded DNA

TBE – Tris Borate EDTA Buffer

TE Buffer – Tris- EDTA Buffer

UV – Ultraviolet

x- SCID - X- severe combined immunodeficiency virus

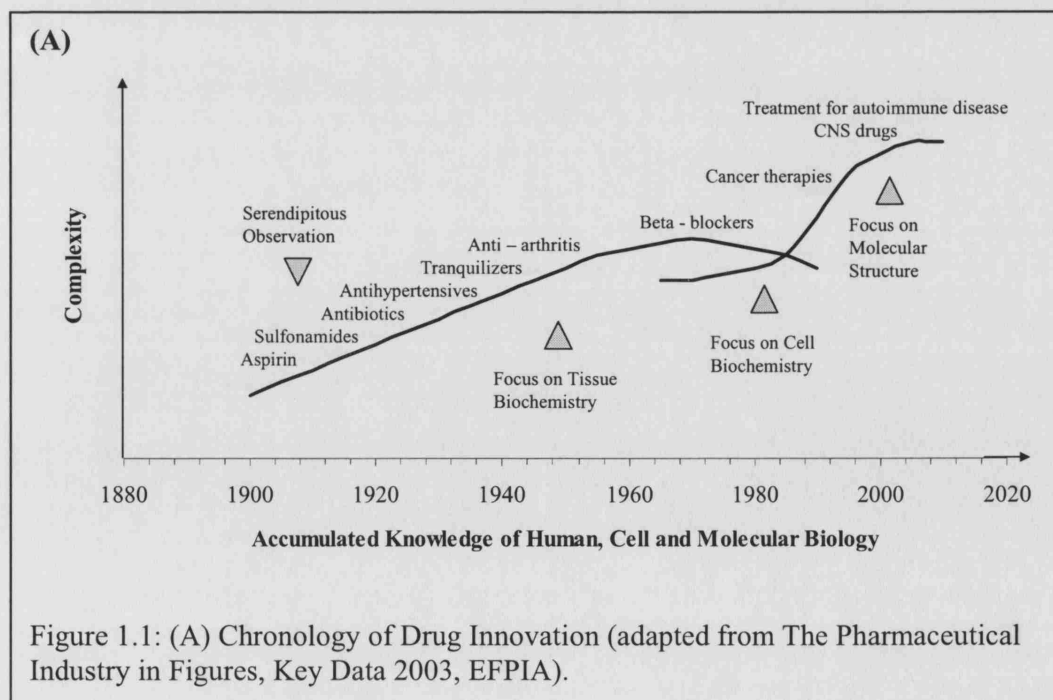
YAC – Yeast Artificial Chromosome

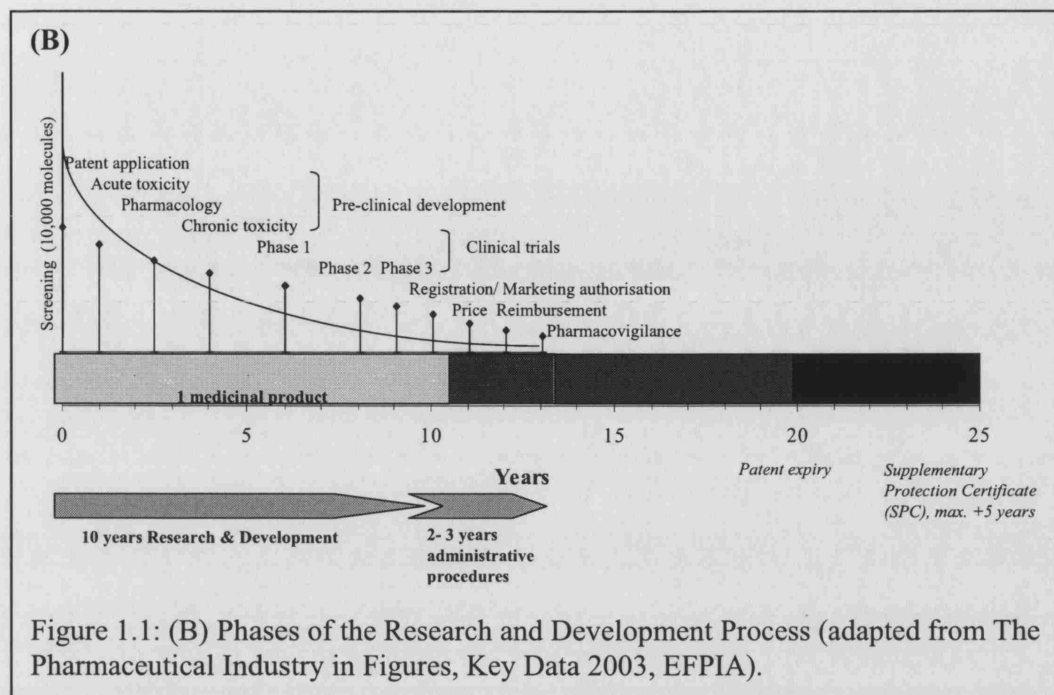
1 PROJECT BACKGROUND

1.1 DNA Vaccination and Gene Therapy

1.1.1 Introduction

The key contribution of the research based pharmaceutical industry to medical progress has been the development of major research findings into innovative treatments that are widely available and accessible (The Pharmaceutical Industry in Figures, Key Data 2003, EFPIA). The research and development of new medicines has seen success against previously fatal diseases and the surge of progress in the treatment of infections such as HIV and cancer. In the past this development evolved from serendipitous breakthroughs such as the discovery of penicillin (Figure 1.1A). However, today's pharmaceutical research is a strictly regulated multi-billion pound industry in which drug discovery, commercialisation and sale can stretch over twenty years (Figure 1.1B).





An element of current critical development is the use of preparations of plasmid DNA for the treatment and prevention of disease. This is an approach to vaccine development that has come to the forefront of medical research in the last ten to fifteen years. The procedure of gene therapy is the delivery of therapeutic DNA constructs to cells, integration into the genome, gene expression and the accession of the job of the host's faulty DNA. An offshoot of this is DNA vaccination where there is no integration of therapeutic DNA into the host genome, but there is transient expression of the delivered genes, which elicits primary (antigenic) and more importantly, secondary (T cell) immune responses (Kelly, 2003; Schleef et al., 2002). Therapies include treatments for cancers (Rubin et al., 1994; Stewart et al., 1999), viral diseases such as AIDS (Semple, 2000; Macgregor et al., 1998; Boyer et al., 1999), conditions such as coronary disease and arteriosclerosis (Rosengart et al., 1999), and crippling genetic disorders such as cystic fibrosis (Porteous et al., 1997).

There are currently over 600 clinical trials either completed, ongoing or pending in the use of gene therapy and DNA vaccines all over the world, Table 1.1 (Journal of Gene Medicine). The growing interest in these treatments consequently requires the large-scale production of pharmaceuticals that meet stringent regulatory standards (Mountain, 2000, Levy et al., 2000). Current standards include the requirement that more than 90 % of the plasmid is in supercoiled form (Butler, 1996). Although small-scale preparations of plasmids less than 30 kb result in a supercoiled yield of 80 - 90 % of the total DNA (Horn et al., 1995; Middaugh et al., 1998), this decreases significantly during large-scale processing and long-term storage as shear, enzymatic and chemical degradation impact on the supercoiled form. For larger plasmids and artificial chromosomes, where not even small-scale preparations yield very high amounts of supercoiled form, damage during large-scale processing and storage is expected to be even more significant.

Diseases	Number of protocols	% of total protocols	Number of patients	% of total patients
<i>Cancer</i>	403	63.4	2392	68.4
<i>Monogenic Diseases</i>	78	12.3	309	8.8
<i>Infectious Diseases</i>	41	6.4	408	11.7
<i>Vascular Diseases</i>	51	8.0	86	2.5
<i>Other</i>	12	1.9	21	0.6
<i>Gene Marking</i>	49	7.7	274	7.8
<i>Healthy Volunteers</i>	2	0.3	6	0.2
Total	636	100.0	3496	100.0

Table 1.1: Clinical protocols by disease (adapted from the electronic Journal of Gene Medicine).

The types of vaccines currently in use include viral vectors (inactivated viruses used to carry the plasmid), naked DNA, and lipid related complexes, as shown in Table 1.2. There are moves towards the use of non-viral vectors as they are less

limited in size and have decreased harmful immune side-effects. Additionally, as the size of plasmids increase there may be greater control and effectiveness through the inclusion of regulatory regions and co-delivery of multiple genes (Mountain, 2000). Plasmid DNA drugs represent a class of materials that is cheap to manufacture, stable and easy to transport at room temperature (Semple, 2000), giving hope of both economic and logistic accessibility to poorer nations.

Vector	Number of protocols	% of total protocols	Number of patients	% of total patients
<i>Adeno-associated virus</i>	15	2.4	36	1.0
<i>Adenovirus</i>	171	27.1	644	18.4
<i>Gene Gun</i>	5	0.8	35	1.0
<i>Herpes Simplex Virus</i>	5	0.8	21	0.6
<i>Lipofectin</i>	77	12.2	619	17.7
<i>Naked Plasmid DNA</i>	70	11.1	123	3.5
<i>Pox Virus</i>	39	6.2	88	2.5
<i>Retrovirus</i>	217	34.4	1757	50.3
<i>RNA Transfer</i>	6	1.0	30	0.9
<i>Others</i>	25	4.0	143	4.1
Total	605	100.0	3496	100.0

Table 1.2: Clinical protocols by vector (adapted from the electronic Journal of Gene Medicine).

One of the primary challenges facing the pharmaceutical industry is the length of product time to market, 12-13 years after the synthesis of a new active substrate, as shown in Figure 1.1B. Pharmaceutical ventures are also notoriously high risk: only 1 - 2 out of 10,000 laboratory synthesised substances become marketable medicines. Another challenge is the costly nature of development and research: the average cost of researching and fully developing a new chemical entity was estimated at £895 million in 2001 (Di Masi, 2001). The appropriate characterisation of complex biologics is vital for the enhanced understanding of bioprocesses as well as cost effective design and development. In the development of DNA vaccines and gene

therapy, the characterisation of these biologic process streams includes the thorough analysis and quantification of plasmid DNA.

Presently accepted methods of plasmid DNA analysis and quantification are both time and labour consuming. Biochemical engineering may provide an important link between the analysis of plasmid process streams at the small-scale, where long and arduous assay techniques are acceptable, and the large-scale where concerns of time and labour are greater and real time analysis may be necessary. In fact, for timely and accurate process design, plasmid analysis at every stage of the process is absolutely critical. Of great importance is the concept of an addressable process for plasmid purification and the development of process analytical techniques (PAT) in which assays (analytical tests) carried out during the process affect the steps taken afterward. This is also quite important in large-scale processes where some production stages take long periods of time and are extremely expensive.

This project focuses on the plasmid purification process and addresses the impact of methods of DNA extraction and purification on the characteristics of the plasmid product. Assays, as well as methodologies for assay development, were designed considering parameters such as accuracy, precision, limit of detection, limit of quantification, specificity, selectivity and robustness. The pharmaceutical research industry urgently needs rapid and cost effective assay methods for use in process monitoring, validation, quality control and regulatory approval by medical councils.

1.1.2 Present Studies and Clinical Trials

The most notable ongoing study involves the pre-conditioning of lung cells affected with cystic fibrosis to help the uptake of therapeutic DNA and extend the length of time of expression. Additionally, research is also ongoing into the use of

gene therapy against Parkinson's disease. In 2002, doctors announced the success of gene therapy treatment against x-Severe Combined Immunodeficiency syndrome (x-SCID) in the UK, but the recent diagnosis of a French patient with leukaemia halted trials against x-SCID in both France and the United States. This occurrence along with the first death of a patient undergoing gene therapy four years ago focused attention on the method of trials and highlighted how much is still unknown about the activities of gene uptake and integration into the host genome.

1.1.3 Comparison of Gene Vectors in Current Use

1.1.3.1 Viral Vectors

Viral vectors are largely the preferred mode of introduction of foreign genes into humans and animals. Gene transfer systems based on de-activated and attenuated viral vectors include adenovirus (AV), retrovirus (RV), lentivirus (LV) and adeno-associated virus (AAV). AV and AAV both have high transfection efficiencies *in-vivo*, efficiencies for LV and RV are lower *in-vivo* although the systems competently transfect *ex-vivo* (Mountain, 2000). They all have prolonged expression but have very small insert-size limits. Additionally, there are safety concerns regarding the strong antigenic immune responses elicited by AV and the maintenance of infectivity of the virus during storage and the immunodeficiency virus origins of LV (Bilbao et al., 1998; Bondoc and Fitzpatrick, 1998; Ory et al., 1996; Zuffery et al., 1997; Wagner, 1998).

1.1.3.2 Lipid Complexes

Lipid complexes are formed when DNA and lipids are mixed and the DNA is condensed and encapsulated by a lipid bi-layer. Cationic liposomes are a

well-established method of delivery of DNA and have substantial advantages over viral delivery systems through their ease of manufacturing, storage and low immunogenicity. Transfection, however, is inefficient and there is short duration of gene expression (Mountain, 2000; Anchordoquy et al., 1998). Additionally, the extreme instability of lipid-DNA complexes in aqueous solution requires that the constituent solutions are mixed and the complexes made just before administration during clinical trials (Allison and Anchordoquy, 2000). Cationic liposomes overcome the unfavourable charge of DNA and condense the macromolecule by decreasing repulsions between DNA segments, either by neutralising phosphate charge or re-orienting water dipoles near DNA surfaces, (Raspaud et al, 1999; Bloomfield, 1998). Uptake into the host cell is thought to occur through endocytosis; however, this mechanism of transfection limits the size of the DNA-lipid complex to 0.1 μm .

1.1.3.3 PLGA Complexes

Poly Lactic-co-Glycolic Acid (PLGA) is a biodegradable material used in the encapsulation of plasmid DNA in spheres of less than 250 μm . Drugs formulated in these polymers are released either by diffusion through the membrane, erosion or a combination of both mechanisms (Wu, 1995). This presents the issues of the biodegradation kinetics of polymer material, biocompatibility and processing of these microspheres (Jain, 2000). PLGA microspheres are too large to enter cells through endocytosis but are effectively taken up by phagocytotic cells such as macrophages, because macrophages are antigen-presenting cells for the immune system, these DNA-polymer microspheres are potentially useful in DNA vaccination (Tabata and Ikada, 1990; Ando et al., 1998; Hedley et al., 1998).

1.1.3.4 Naked DNA

The main benefit of naked DNA is its easy manufacture, storage, process control, monitoring and quality control (Durland and Eastman, 1998; Ledley, 1995). Although transfection is inefficient *ex-vivo*, some studies have shown that gene transfer is surprisingly efficient, especially following injection into muscle or skin (Wolff et al., 1992; Hengge et al., 1995). The mechanism of DNA uptake and subsequent expression or antigen presentation *in-vivo*, is largely unknown (Semple, 2000) and its expression is transient and can last from hours to several days (Mountain, 2000). Naked DNA does not provoke specific immune response, but when produced in bacteria, unmethylated CpG sequences have been shown to elicit a stimulatory immune and inflammatory response in animals (Mountain, 2000). This response is useful for DNA vaccination as the body's immune system is aroused; however, this is a disadvantage in the treatment of chronic diseases where constant inflammation may put patient welfare at risk.

1.1.3.5 Particle and Energy Mediated Methods of Gene Delivery

Other methods of gene delivery include particle bombardment whereby a gene gun may be used for the *in vivo* transformation of cells or organisms for the purpose of gene therapy and DNA vaccination. The gun uses bombardment where DNA- or RNA-coated gold particles are loaded into the gun and a low pressure helium pulse delivers the coated gold particles into virtually any target cell or tissue. This capability is particularly useful for research efforts to broaden understanding in gene therapy, especially for cancer biology and wound healing (Davidson et al., 2000). One example of the gene gun is the Helios gun produced by BioRad which is reported as requiring only microgram amounts of DNA by the manufacturers (www.bio-rad.com).

Both humoral (antibody-based defenses in blood and lymph systems) and cell-mediated immunities have been demonstrated in animals using this technology (Tang, 1992; Qiu P, 1996).

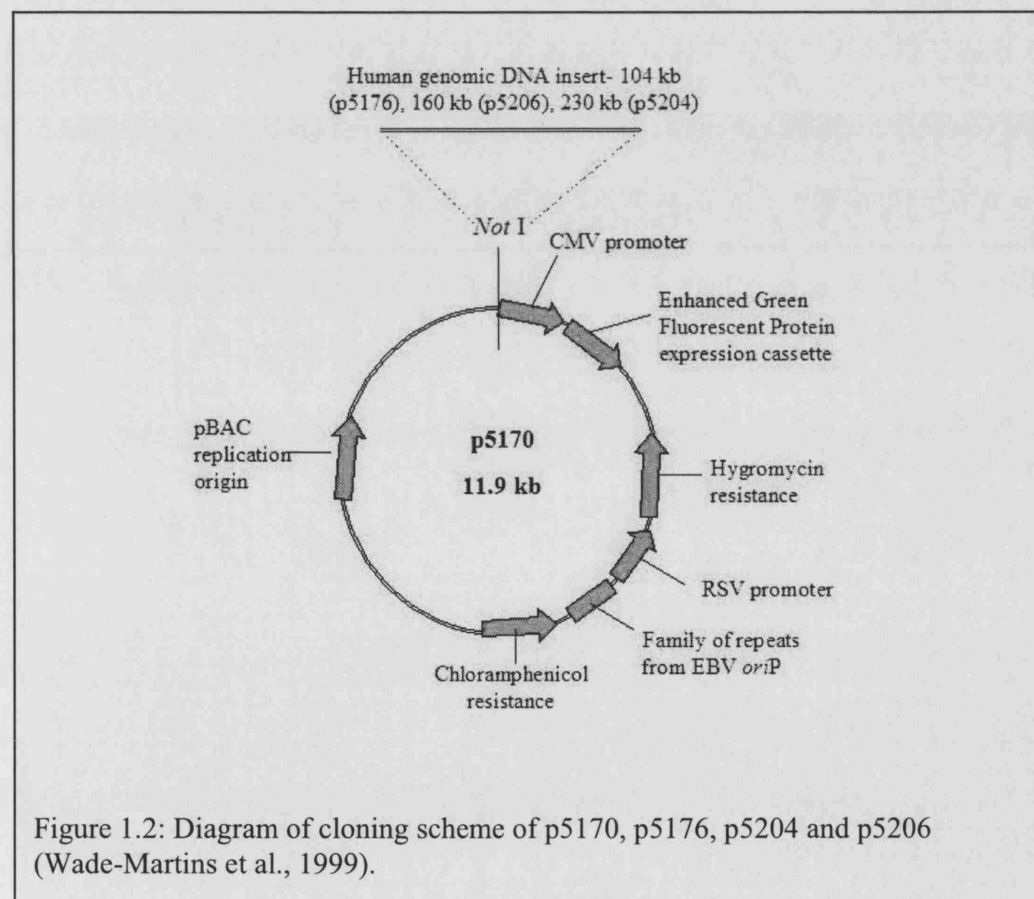
Another exciting method is the use of acoustically active microbubbles for the ultrasound mediated delivery of nucleotides. In vitro studies using perfluorocarbon-exposed sonicated dextrose albumin (PESDA) microbubbles have shown binding to oligonucleotides and that ultrasound can be used to deposit these nucleotides. In addition, in vitro studies show that drug release from microspheres is dependent on ultrasound transmission frequency as well as pulsed or continuous application (Porter and Xie, 2001).

1.2 Plasmids and the *E. coli* cell

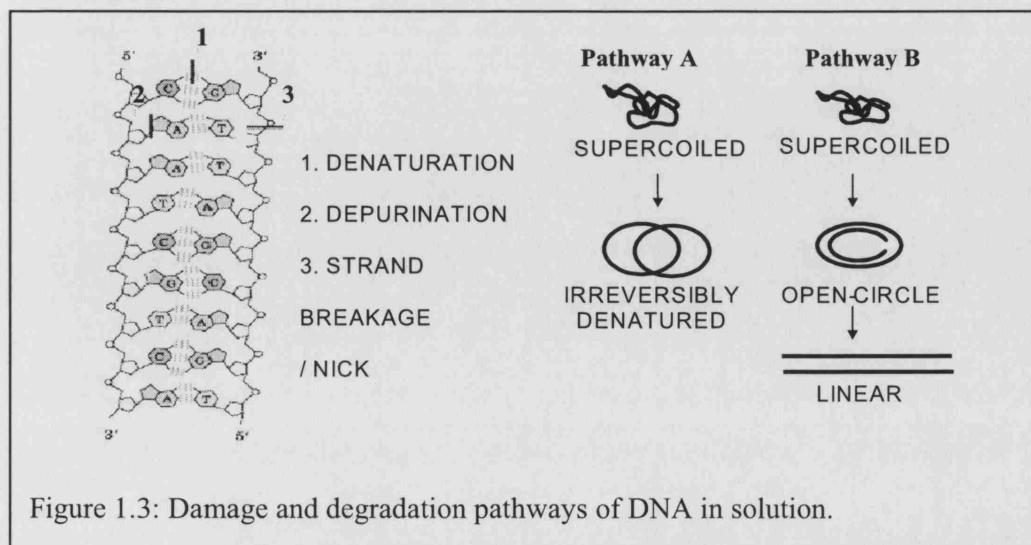
1.2.1 Plasmids and DNA topology

Plasmid DNA based expression systems comprise of three major genetic components. These include the prokaryotic plasmid vector, eukaryotic regulatory elements that control the location, level and duration of therapeutic protein expression in host cells, and the gene or genes that code for the therapeutic or antigenic proteins (Durland and Eastman, 1998). Through the increase in size of plasmid DNA there may be more effective targeting and control in gene therapy due to the inclusion of multiple genes; naked DNA vaccines can be developed to be polyvalent, and so able to target more than one epitope (Semple, 2000). Figure 1.2 shows the cloning scheme of the family of bacterial artificial constructs (BACs) used in this project. The vector contains a human genomic insert as well as the selective marker for chloramphenicol resistance, the Epstein-Barr virus family of repeats which promote the maintenance of plasmid as episomes in mammalian cells, and the promoter of the cytomegalus virus

(CMV) (Wade-Martins et al., 1999). The BAC vectors used in this project were 116, 172 and 242 kb containing inserts of 104, 160 and 230 kb, respectively. They all exist as one copy per bacterial cell as their origin of replication is based on the F1 plasmid. They are thus, considerably larger than the other plasmids used in this project which were pSV β which is 6.9 kb in size and is a pUC based plasmid with high copy number (~ 500 copies per cell), pQR150 which is 20 kb in size (Jackson et al., 1995) and also exists in high numbers in the bacterial cell; the plasmids contain inserts for ampicillin and kanamycin resistance, respectively. The plasmid maps for all plasmids used are shown in the Methods and Materials.



Similar to peptides and proteins, nucleic acids exhibit not only primary structure but secondary and tertiary structure, which facilitate biological activity. Any chemical instability affecting nucleic acids may result in physical instability leading to degradation, denaturation, aggregation or precipitation (Pogocki and Schoneich, 2000). One of the major factors affecting transfection efficiency is the topology of plasmid DNA. Changes in DNA topology result from physical instability leading to changes in tertiary structure: for example, DNA changes from a covalently closed, tightly supercoiled formation, to the open-circular isoform. Additionally, further breakage of the sugar phosphate backbone produces the linear isoform, as shown in Figure 1.3. Studies have shown the efficiency of transfection to vary depending on the conformation, where the supercoiled form is most effective and the linear form is the least effective (Cherng et al., 1999); this is perhaps partly due to the susceptibility of linear DNA to exonuclease digestion (Weintraub et al., 1986). Monitoring the percentage of supercoiled DNA is notably one of the process requirements of FDA licensed materials; this is expanded in section 1.3. Figure 1.4 shows the form of 6 kb supercoiled plasmids. In nature, the compaction of DNA through supercoiling allows hundreds of such plasmids to fit into a single *E. coli* cell



with a diameter and length of 0.5 and 2 μm , respectively.

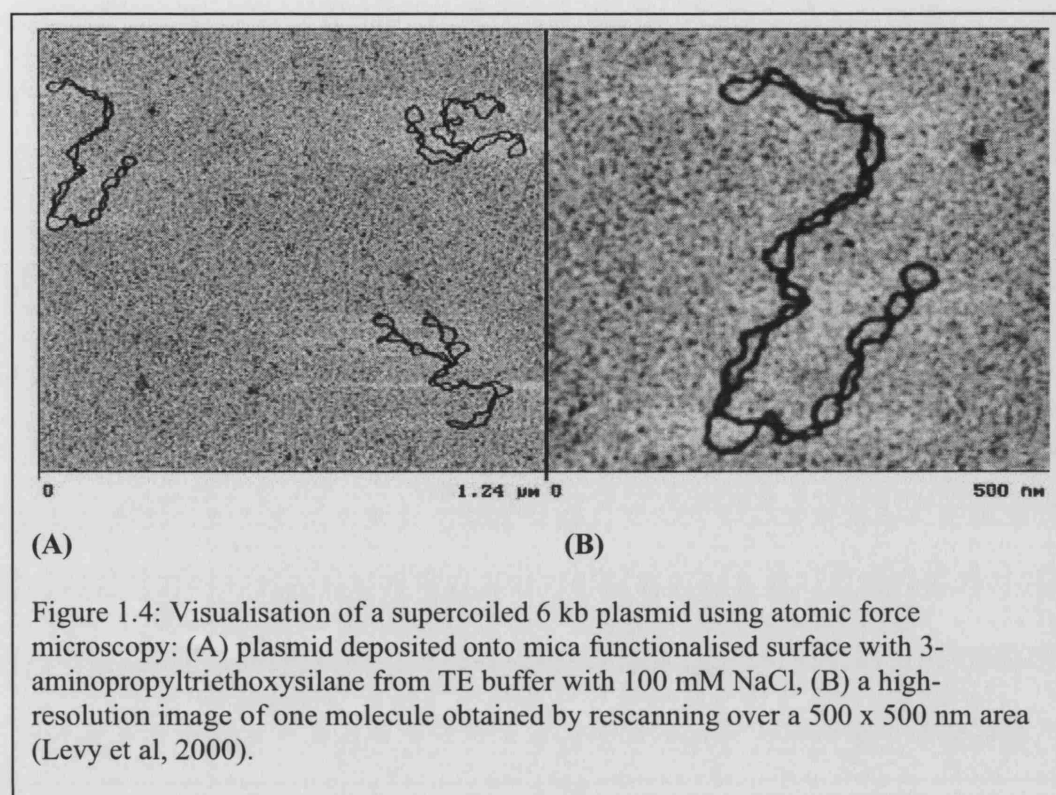
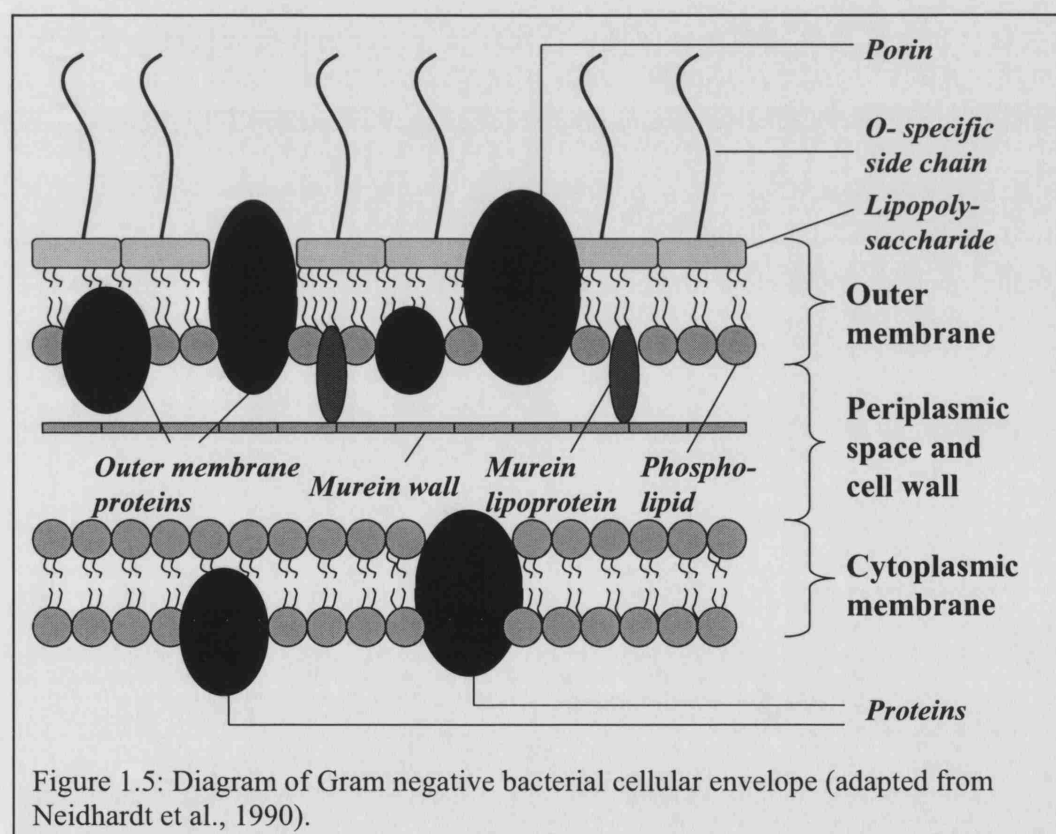


Figure 1.4: Visualisation of a supercoiled 6 kb plasmid using atomic force microscopy: (A) plasmid deposited onto mica functionalised surface with 3-aminopropyltriethoxysilane from TE buffer with 100 mM NaCl, (B) a high-resolution image of one molecule obtained by rescanning over a 500 x 500 nm area (Levy et al, 2000).

1.2.2 The *E. coli* cell

The *E. coli* bacterium is a well-characterised organism used extensively in microbiology. This prokaryote is rod shaped of the average dimensions mentioned in the previous section. A consequence of the small size of the bacterium is a large surface to volume ratio which affords the rapid transfer of nutrients and waste materials and hence, a high metabolic rate and rapid growth. Figure 1.5 shows the cellular membrane and wall of a gram-negative bacterium. This class of bacterium has evolved a special way of protecting its cytoplasmic membrane; the outer membrane has the ability to resist damaging chemicals (Neidhardt et al, 1990; Freifelder, 1987). Whilst the inner membrane resembles other phospholipid bilayers, the outer membrane utilises lipopolysaccharides (LPS), a complex molecule not found

elsewhere in nature. The lipopolysaccharide consists of three parts: (i) a glycolipid called *lipid A*, (ii) a short series of sugars called the *core* and (iii) a carbohydrate chain up to forty sugars in length called the *O-specific side chain*. It is this side chain that has been found to play a role in certain types of antibiotic resistance as well as resistance to bile salts. Importantly, lipopolysaccharides, also known as endotoxins, serve to advertise the presence of bacteria in animal tissues. The lipid A component, in small doses, elicits fever and an immune response; in large doses, it can lead to shock and death. Thus, there is strict regulation regarding the presence of endotoxins in bacterial derived pharmaceuticals, as further discussed in section 1.3.2.



The *E. coli* bacterial membrane excludes large and small hydrophobic molecules and allows entry of small hydrophilic molecules (less than 600-700 Da) through porins. Larger hydrophilic molecules such as iron chelates and vitamin B₁₂

are allowed entry through specially dedicated mechanisms utilising proteins. The dual membrane system of the Gram-negative bacterium creates a periplasmic space, which makes up 20-40 % of the cell volume. It contains the murein layer and a gel-like solution of proteins. These include binding proteins that help adsorb sugars, amino acids, vitamins and ions, degradative enzymes such as phosphatases, proteases and Endonuclease I; there are also detoxifying enzymes such as β -lactamases which deactivates penicillin and the aminoglycoside-phosphorylating enzyme. The periplasm has the same osmolarity as the cytoplasm and this appears to be partially regulated by a class of compounds called membrane derived oligosaccharides. It has been shown that the synthesis of these compounds can be sensitive to certain conditions of growth (Neidhardt et al, 1990). The structure and mechanisms of the bacterial membrane has important implications for assay development, as discussed in later chapters.

Besides the mechanisms of action of the bacterial membrane, another important aspect of the *E. coli* bacterium that should be considered is its general composition. The cell is typically composed of, on a dry weight basis, proteins (55%), RNA (20%), lipids (12%) and DNA (5%), see Table 1.3. Other constituents include murein, glycogen, metabolites, vitamins and inorganic ions. The weight of one cell is around 7.5×10^{-13} g with about 70% of this mass being water. Thus, when bacterial cells are lysed a very complex solution is released of which plasmid DNA is only a very small part, 2-3% of dry cell weight (Ayazi Shamlou, 2003; Neidhardt et al., 1990). Plasmid extraction from this mixture is detailed in Methods and Materials-Chapter 2. Lysis with alkaline and detergent serves to rupture cells and denature DNA and further acidification renatures plasmid DNA and precipitates chromosomal DNA, proteins and cell debris (Birnhoim and Doly, 1979). This lysis is the most critical

downstream processing step in the production of plasmid as sheared forms of host cell chromosomal DNA are very difficult to separate from plasmid product. Lysed cells also exhibit unique rheological properties to which large-scale process machinery must be adapted. Additionally, RNA can also be degraded into short fragments of a similar size to plasmid DNA and endotoxins may be difficult to detect or separate by electrophoresis and related methods as they have mass charge ratios similar to DNA (Kelly, 2003; Varley et al., 1999). Cell constituents other than plasmid DNA product may be seen as process stream contaminants which may detrimentally impact the quality of the plasmid product. They may also present a load to purification methods such as chromatography and so decrease process efficiency and yield.

Cell constituent	% of cellular dry weight
Proteins	55
RNA	20
Chromosomal DNA	3
Plasmid DNA	2
Lipids	12
Others	8

Table 1.3: Constituents of the *E. coli* cell (adapted from Ayazi Shamlou, 2003).

1.3 Regulation and Monitoring of Plasmid DNA Pharmaceuticals

1.3.1 Current Standards of Plasmid Pharmaceutical Processing

The current production and characterisation processes for plasmid- based drugs have been largely adapted from large-scale protein manufacture and purification processes and those of traditional molecular biology. In the late 1990s changes in the regulation of biologics by the FDA streamlined the review procedures required for new product approvals and manufacturing changes (Durland and Eastman, 1998), foremost of these changes was the definition of “well-characterised” biotechnology

products (Weschler, 1996). Considerations for investigational new drug (IND) applications include three stages: (i) the characterisation of plasmid products by direct sequence and restriction enzyme analysis, (ii) the testing of master and working cell banks to ensure freedom from contamination by bacteriophages and other agents and (iii) the maintenance of specific characteristics such as antibiotic resistance. For the bulk plasmid product, standard assays of specificity and sensitivity should be used and validated using known reference materials and spiking experiences (Butler, 1996). Validation is defined by the FDA as establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its pre-determined specifications and quality attributes. Within later chapters, the term validation is extended to determining that the assay methods developed can consistently measure quality attributes within pre-determined parameters. A wider extension of the idea that the assays developed are fit for the purpose of reliable and reproducible measurement.

Recently, Process Analytical Techniques (PAT) have been publicised by the FDA as the new system for the validation of pharmaceutical processes (www.fda.gov/cder/OPS/PAT). PAT are systems for the analysis and control of manufacturing processes based on measurements of critical quality parameters and performance attributes to ensure acceptable end quality of pharmaceutical products. Thus, validation is designed into the purification process with analysers and other technologies to perform not only assays on the product and process stream but also to self-validate and perform routine checks; for example, the calibration of analysers and assessing the suitability of system and sample streams. Quality assurance is continuous and this represents a paradigm shift in validation. PAT not only allows on-line and in-line measurements but also at-line testing where assays are designed to

be rapidly performed on the manufacturing site significantly decreasing time delays associated with off-site testing. The impact on industry will be to provide a means by which shorten the time between processing and quality assurance and hence decrease production times and increase the overall efficiency of the industrial process.

1.3.2 Validation and Quality Control

FDA guidelines state that DNA content in the bulk plasmid product should be validated by A260/A280 measurements and agarose gel electrophoresis methods. RNA contamination of the plasmid process stream should also be validated by these methods. Additionally, homogeneity of plasmid size and DNA isoforms should also be monitored. Potency assays must measure the level of production and immunogenicity of the gene product. Additionally, expression of the product may be determined by transfection studies. Protein, endotoxin and bacterial genomic DNA levels should be monitored and validated; this is all shown in Table 1.4 (Butler, 1996; Ferreira et al., 2000).

Impurity	Recommended Assay	Approval Specifications
<i>Proteins</i>	Bicinchinoic assay	Undetectable
<i>Genomic DNA</i>	Agarose gel electrophoresis	Undetectable
	Southern Blot	< 0.01 µg/ µg plasmid
<i>RNA Transfer</i>	Agarose gel electrophoresis	Undetectable
<i>Endotoxin</i>	<i>Lymulus amebocyte</i> lysate (LAL) assay	< 0.1 Endotoxin Unit/ µg plasmid
<i>Plasmid isoforms (relaxed, linear and denatured)</i>	Agarose gel electrophoresis	< 5%
<i>Biological activity and identity</i>	Restriction nucleases	Coherent fragments with plasmid restriction map
	Agarose gel electrophoresis	Expected migration from size and supercoiling
	Transformation efficiency	Comparable with plasmid standards

Table 1.4: The principle approval specifications and recommended assays for assessing purity, safety and potency of DNA preparations for gene therapy and DNA vaccination (Ferreira et al, 2000).

The assays developed to augment or replace established methods of monitoring and validating plasmid process streams must fulfil the following characteristics, defined according to general FDA (www.fda.gov/cder) and IUPAC guidelines:

- i. *Identity*: The assay should establish the identity of the drug product described, including an evaluation of specificity and sensitivity or selectivity. Purity and potency should also be considered and validated.
- ii. *Accuracy*: The closeness of the agreement between a test result and the accepted reference value.
- iii. *Precision*: The closeness of agreement between independent test results obtained under stipulated conditions (different days and times), precision depends on random errors and not systemic error or bias, which should be

considered separately. Repeatability and Reproducibility are other closely related assay characteristics.

- iv. *Limit of detection*: This is the lowest content that can be measured with any statistical certainty. This must be considered with reference to blank samples.
- v. *Quantitation*: This is usually determined by a calibration curve, notably the limit of quantitation is equal to or greater than the lowest concentration point of the calibration curve. Quantification limits are the performance characteristics of the assay to adequately quantify levels of the analyte within a particular confidence range.
- vi. *Specificity*: The ability of the test to measure only what it is intended to measure.
- vii. *Selectivity*: The ability of an analytical test to discern accurately and specifically the analyte of interest among other components in a sample matrix under test conditions. In analysis, the extent to which contaminants interfere with test results may be determined as a co-efficient or constant.
- viii. *Robustness*: This is seen as the capacity of an analytical procedure to remain unaffected by variations in method parameters and is an indication of its reliability in normal usage.
- ix. *Ruggedness*: This is an intra-laboratory study analysing the behaviour of small changes in operating conditions to the analytical procedure. These changes should model those likely to arise in different test environments.

The suggested tolerance for validation parameters is coefficient of variation (CV) < 15% for bioanalytical methods, however, this tolerance is reduced greatly for industrial pharmaceutical procedures to < 2% (Lindholm et al., 2003).

1.4 Monitoring of Process Streams of Plasmid and BAC DNA

1.4.1 Current Methods of Assaying DNA Quality

1.4.1.1 Electrophoresis

Presently accepted methods of DNA analysis include agarose gel electrophoresis, capillary electrophoresis and chromatographic techniques, which are either time and labour consuming or use significant amounts of plasmid product (Levy et al., 2000). Nucleic acid electrophoresis involves the separation of DNA and RNA based on mass charge ratio and size through a porous gel matrix suffused with ion containing solution using an electric field (Westermeyer, 2001). Nucleic acids are negatively charged due to their ionized phosphate groups. As the size of nucleic acids increases, charge also increases so the mass-charge ratio remains constant. Thus electrophoretic separation of these compounds is almost entirely based on size. This is achieved with the sieving effect of the gel matrix. It is not a very accurate method as there is a reported error of 10-20 % (Projan et al., 1983; Levy et al., 1999). These methods also pose other limitations, although agarose gel electrophoresis is the recommended assay for assessing plasmid isoforms (Butler et al., 1996), the upper size limit of DNA separation is 30-50 kb. Thus, to analyse larger plasmids and constructs, pulse field gel electrophoresis (PFGE) may have to be used which is very expensive, labour intensive and usually requires a run-time of more than 12 hours. However, PFGE enables the resolution and analysis of pieces of DNA well over 10,000 kb. The basic principle of DNA separation during PFGE is that while DNA above 30-50 kb moves in a diffuse band during conventional agarose gel electrophoresis, if the DNA is made to change direction by reorienting the electric field the smaller fragments within this diffuse band will move more quickly in the

new direction leaving larger fragments behind; fragments essentially “wobble” through the pores of the agarose gel. The resolution of DNA fragments depends on parameters such as the mass of the DNA fragment, field strength, pulse time, reorientation angle, temperature, buffers and percentage agarose used (Flickinger et al., 1999; Anand and Southern, 1990). The DNA is visualised using binding dyes which fluoresce under UV light, such as ethidium bromide. The separation of high molecular weight DNA through PFGE has also become a tool of purification with subsequent digestion of the gel by β -agarase (Compton et al., 1999) and by the trapping of open-circular DNA in agarose (Cole and Akerman, 2000; Akerman and Cole, 2002; Cole and Tellez, 2002). Recent work has shown that the separation of DNA molecules in excess of 100 kb in less 1 minute with the use of scaled down electrophoresis equipment and pulsed field electrophoresis preceded by entropic focusing (Bakajin et al., 2001).

1.4.1.2 Capillary Electrophoresis and Capillary Gel Electrophoresis

In comparison, capillary electrophoresis (CE) is able to purify samples in a rapid (within half an hour), automatable format. It is a collection of techniques where high voltages are applied across a buffer filled capillary, to achieve separation based on size and charge, followed by UV absorbance detection. Heat dissipation across the wall of the capillary provides higher voltages and faster separations allowing for on-line analysis and feedback. For the analysis of large bio-molecules such as DNA with similar mass to charge ratios, the separation is carried out in a polymer filled capillary in order to increase resolution, a technique known as capillary gel

electrophoresis (CGE). This polymer is usually cellulose derived and the capillary is refillable. In CGE a sieving effect occurs where small molecules migrate quickly and the larger molecules become entangled in the matrix and their movement is inhibited. This is similar to, though distinct from, gel permeation chromatography where large molecules are completely excluded from the matrix and elute first and the smaller molecules that move into and through the matrix take longer to elute (Shieh et al., 1998; Weinberger, 2000). The CGE technique was first developed in the late 1980s for the separation of DNA and is successfully able to separate plasmid DNA as well as seeing extensive analytical use in the Human Genome Project (Kasper et al., 1988; Altria, 1999). CGE and its offshoot high-performance capillary electrophoresis (HPCE), however, remain very efficient and quick microseparation techniques. They are expensive to implement and unsuitable for pilot plant and manufacturing scale. Recently, CGE has been used to determine plasmid topologies and correlate these with transfection efficiencies (Walther et al., 2003). The CGE analysis revealed that stable storage conditions at -80°C prevented an increase in open circular (oc) plasmid, preserving the covalently closed circular (ccc) form, which is sought for efficient gene transfer. By contrast, long-term storage of plasmid DNA at 4°C lead to the rapid decline of the ccc form and the increase of oc and linear DNA molecules. The use of naked DNA stored for 1, 2, or 13 months at -80°C showed similar *in vivo* transfer efficiencies by jet-injection. Therefore, it was determined by Walther et al, 2003, that analysis of plasmids by CGE allowed the reliable determination of integrity and distribution of the topology of the DNA by quantitative means.

1.4.1.3 Chromatography

Chromatography is the most popular method for the large-scale purification of supercoiled DNA. Chromatography is the resolution of a chemical mixture into its component compounds by passing it through a system that retards each compound to a varying degree. The retarding system can be a surface adsorbant, such as silica, alumina, cellulose, or charcoal, capable of reversibly adsorbing the compounds or a porous particle capable of admitting molecules of particular size as in size exclusion chromatography.

In column ion chromatography the adsorbant is packed into a column and a solution of the mixture is added at the top. An appropriate solvent is passed through the column, washing, or eluting, the compounds down the column. A polar substance that is adsorbed very tightly to the surface will be efficiently retarded by the column, while a nonpolar substance will elute (dissolve in the solvent) very rapidly. By varying the nature of the solid adsorbant and the eluting solvent, a wide variety of resolutions, even of very similar substances, can be carried out. For compounds that can exist as anions such as the negatively charged DNA or RNA, anion-exchange chromatography can be used to separate them from neutral or oppositely charged compounds. The mixture is added to a column packed with a porous, insoluble resin which has a positively charged (cationic) group attached to it and an unattached, negatively charged (anionic) counterion. An anion from the mixture will exchange with the negative counterion of the resin and will be retarded while neutral and cationic substances are not affected. Fractions of differing charges may be unbound from the resin and eluted using a solvent with increasing ionic gradient.

Size exclusion chromatography (SEC) is the separation of mixtures based on the molecular size of the components. Separation is achieved by the differential exclusion or inclusion, within the packing particles, of the sample molecules as they pass through a porous-particle stationary phase. Due to its relative non- interaction with the sample in comparison to anion exchange chromatography, SEC is seen as a more gentle method and enables the high retention of biomolecular enzymatic activity while separating multimers that are not easily distinguished by other chromatographic methods. In aqueous systems, SEC is referred to as gel filtration chromatography (GFC).

Anion exchange and size exclusion chromatography are both used at lab and industrial scale. This method has the advantage of being easily scaleable but is hindered by the low performance of sorbents currently on the market (Ferreira et al., 2000). These sorbents were mostly designed with protein separation in mind. High performance liquid chromatography (HPLC) is a faster method of separating DNA. It is similar to CE in its ease of use, high resolution, speed, on-line detection and automation capability. There are several modes of HPLC, varying with the stationary phase used. The detectors used also vary, for identifying DNA, UV absorbance or fluorescent dyes may be used, as in standard chromatography. HPLC is carried out using column packing particles of 2-5 μm in diameter. Due to the size of these particles high pressures (5-10 MPa) must be used to achieve liquid flowrates of typically 1-5 ml min^{-1} . At larger scale, pressure and resolution are sacrificed, making HPLC a technique suitable for small-scale analytical applications. Chromatography column cleaning in place is difficult and harsh treatments may be necessary to clean and regenerate, there is also a high cost so the columns must be re-used (Doran, 1995; Shelley et al., 1996). There is the possibility that large DNA molecules may be

damaged by the high forces exerted in a HPLC column, making it an unsuitable technique for use on large plasmids and artificial chromosomes. Gels used for chromatography are compressible and cannot withstand high HPLC pressure, and techniques developed that combine HPLC and CE, such as capillary electrochromatography (CEC), are used mainly for the resolution of non-ionic compounds. Although charged solutes have proved problematic with CEC, there have been reports of separation of nucleotides by Altria, 1999 and Helboe and Hansen, 1999.

Recent studies conducted by Molloy et al, 2004, have utilised anion- exchange high -performance liquid chromatography (AEC) and an agarose gel electrophoresis (AGE)- based method in order to determine the plasmid DNA topology for a 6.5 kb pUC based 'DNA vaccine' plasmid. These methods are directly compared, limitations addressed and techniques for overcoming these limitations described.

1.4.2 Methods for Assessing Contaminants

Blot hybridization (Northern and Southern) in combination with agarose gel electrophoresis is a generally accepted method for determining host DNA and RNA contamination in plasmid DNA product. More recently methods have been developed at the FDA Centre for Biological Evaluation and Research (CBER) where quantitative assays have been developed to assess the biological activity of residual host DNA and RNA (<http://www.fda.gov/cber>). Nucleic acid contamination has been considered to pose a risk to vaccine recipients with both infectious and oncogenic potential. Quantitative PCR (Q-PCR) assays have been developed for viral, bacterial and mammalian RNA and DNA (Maudru and Peden, 1998; Richard et al, 2003).

As with DNA host cell contamination, patient safety also demands that protein contamination be reduced to the lowest levels practicable. Host cell protein contamination may cause adverse immune reactions within recipients of plasmid DNA therapies. In order to detect possible protein contamination silver stained gel should be used in preference to Coomassie blue, and immunologic techniques such as enzyme-linked immunosorbent assay (ELISA) or Western blot should also be utilised (Butler, 1996). Of special importance is the quantification of endotoxins, the LAL assay and variations of this method are the most popular techniques for detecting these substances. Table 1.4 (in section 1.3.2) shows the principle approval specifications for the lot release of plasmid as a pharmaceutical product.

1.5 Fluorimetry and Fluorochromes

1.5.1 Introduction

Applications of fluorescence in the field of nucleic acid chemistry started in the late 1950s, with the first reports of the native fluorescence of adenine and guanine solutions (Duggan et al., 1957). Fluorescence may be described as the emission of a photon from an excited molecule *via* the excitation of the molecule and the absorption of a photon. Fluorescence has already been used with nucleic acids to provide information on flexibility, torsion dynamics, helical structure, deformation due to intercalation, photocleavage and carcinogenesis (Valeur, 2002). Photospectroscopy, using UV absorbance at 260 nm, is the most commonly utilised method for nucleic acid detection but it is notoriously imprecise; absorbance methods cannot distinguish between RNA and double stranded and single stranded DNA forms (Rompannen et al., 2000). A value of A_{260} of 0.1 corresponds to a $5 \mu\text{g mL}^{-1}$ dsDNA solution which also corresponds to a $4 \mu\text{g mL}^{-1}$ RNA. The use of fluorescence stains in detection and

quantitation of nucleic acids is common due to ease of use and the most popular method of detection using agarose gel electrophoresis utilises ethidium bromide, an intercalating dye, to bind to DNA in agarose. Newer nucleic acid dyes still provide ease of use but offer greater sensitivity and large dynamic range, PicoGreen is one of this new generation of nucleic acid stains produced by Molecular Probes (Leiden, Netherlands).

1.5.2 Nucleic Acid Stains: Properties and Mechanisms of Binding

Classic nucleic acid stains may be divided into three types: (i) intercalating dyes, which slide between the base pairs in DNA such as ethidium bromide, (ii) minor-groove binders such as Hoescht 33258 which bind to the minor groove of DNA and (iii) miscellaneous stains such as acridine orange which have special properties such as cell permeability. The newer dyes, such as PicoGreen, belong to a class of proprietary cyanine dyes. This class may be further divided into premiere ultra-sensitive dyes, cell penetrating and cell impermeant dyes and chemically reactive dyes that form bioconjugates (Haughland, 2001). These dyes all fluoresce at varying wavelengths between 300 and 650 nm. Additionally, the proprietary cyanine dyes, produced by Molecular Probes, offer the following: (i) high molar absorptivity, with extinction coefficients typically $>50,000 \text{ cm}^{-1}\text{M}^{-1}$ at visible wavelengths, (ii) very low intrinsic fluorescence, large fluorescence enhancements (often over 1000-fold) upon binding to nucleic acids, and (iii) moderate to very high affinity for nucleic acids, with little or no staining of other biopolymers (Haughland, 2001). As yet, the mechanism of binding of PicoGreen and related stains is undiscovered but it is thought that fluorescence occurs with the de-excitation of the dye molecule bound

to DNA through intercalation and surface groove interactions, (Haughland, 2001; Singer et al., 1997).

Ethidium bromide (2,7- diamino-10-ethyl-9-phenylphenanthridinium bromide, EtBr), Figure 1.6, is a cell impermeant phenanthridinium intercalator. It can be excited with mercury or xenon arc lamps or with an argon-ion laser, making it suitable for fluorescence microscopy, confocal laser scanning microscopy, flow cytometry and fluorometry. This dye binds with little or no sequence preference at a stoichiometry of one dye per 4–5 base pairs of DNA (Waring, 1965). Excitation of the EtBr-DNA complex may result in photobleaching (due to irreversible damage of the dye) and single-strand DNA breaks (Krishnamurthy et al., 1990). EtBr also binds to RNA, and once EtBr is bound to nucleic acids, fluorescence is enhanced ~20-30 times: excitation maxima are shifted ~30–40 nm to the red and their emission maxima are shifted ~15 nm to the blue (Ardnt- Jovin and Jovin, 1989). The ethidium bromide molecule may be seen to be aromatic in nature and is known as a cyclic conjugated system (Valeur, 2002). These aromatic rings intercalate and bind to the base pairs in DNA through hydrogen bonding. The diffuse electron field of the dye complex becomes excited by photons in its bound state, stores this energy and rapidly releases it as fluorescence.

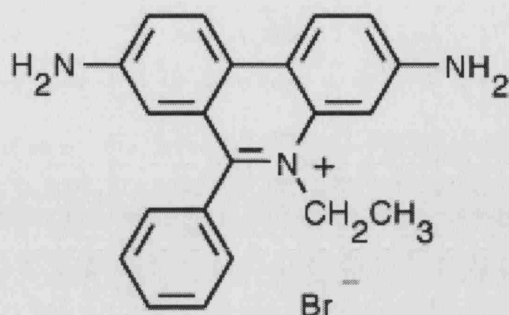


Figure 1.6: Chemical Structure of Ethidium Bromide.

1.5.3 Use of Nucleic Acid stains within the Project

1.5.3.1 PicoGreen

PicoGreen is an ultrasensitive cyanine based DNA binding dye capable of detecting from picogram to milligram quantities of double stranded DNA (dsDNA). It has been used extensively during this project due to its sensitivity, wide working range and ease and rapidity of use. The dye binds to dsDNA with high specificity and also shows binding to single stranded DNA, but at one thousandth of the fluorescence emission signal of dsDNA binding. The PicoGreen assay is greater than 10,000 times more sensitive than conventional UV absorbance measurements at 260 nm, at least 400 times as sensitive as the Hoechst 33258 dye-based assay (which shows significant AT selectivity) and is more sensitive than assays based on YO-PRO-1 and YOYO-1 dyes (Johnson et al., 1992). The dye is well characterised and stable over a wide pH range (Singer et al., 1997). Its excitation and emission wavelengths are 480 and 520 nm respectively. The dye shows high selectivity and robustness with linear response in the presence of several compounds found in nucleic acid preparations such as urea, detergents, proteins and agarose (Haughland et al., 1996; Singer et al.,

1997). In the characterisation of PicoGreen by Singer et al., 1997 and within the manufacturer's data (<http://www.probes.com/handbook>), there are defined concentrations in which signal depression has been shown to occur with compounds commonly found within nucleic acid preparations. These are shown in Table 1.5. Within the experimental protocols care was taken to ensure that these possible contaminants were diluted well below levels reported to cause interference with the PicoGreen signal. This is further described in Chapter 3 (for protein contamination) and Chapter 6 (for SDS and acetate contamination). Prior investigations have focused on the use of PicoGreen as a method of quantitation for plasmid, viral and mammalian DNA (Ribiero et al., 2000; Murakami and MacCaman, 1999; Charlton et al., 1999). However, recent studies have seen the use of the dye in combination with heat treatment (Levy et al., 2000) and to study the DNA type and content of whole cells (Batel et al., 1999). It is, in short, a very good reagent for DNA quality and quantitation assay development within pure and crude solutions, its use is a continuation of previous work on assay development at University College London, UK (Levy et al., 2000).

Compound	Concentration	% Signal Change *
Salts		
Ammonium acetate	50 mM	3% decrease
Sodium acetate	30 mM	3% increase
Sodium chloride	200 mM	30% decrease
Zinc chloride	5 mM	8% decrease
Magnesium chloride	50 mM	33% decrease
Urea	2 M	9% increase
Organic Solvents		
Phenol	0.1%	13% increase
Ethanol	10%	12% increase
Chloroform	2%	14% increase
Detergents		
Sodium dodecyl sulfate (SDS)	0.01%	1% decrease
Triton X-100	0.1%	7% increase
Proteins		
Bovine serum albumin (BSA)	2%	16% decrease
IgG	0.1%	19% increase
Other Compounds		
Poly(ethylene glycol)	2%	8% increase
Agarose	0.1%	4% increase
* The compounds were incubated at the indicated concentrations with PicoGreen reagent in the presence of 500 ng/mL calf thymus DNA. All samples were assayed in a final volume of 200 μ L in 96-well microplates using a CytoFluor microplate reader. Samples were excited at 485 nm, and the fluorescence intensity was measured at 520 nm.		

Table 1.5: Effects of Contaminants on the PicoGreen Assay

Effects of several compounds that commonly contaminate nucleic acid preparations on the signal intensity of the PicoGreen dsDNA quantitation assay. (From the web edition of *The Handbook — A Guide to Fluorescent Probes and Labeling*, 10th edition, www.probes.com/handbook)

The beneficial aspects of PicoGreen and closely related dyes are detailed below (Haughland, 2001):

Sensitivity

The PicoGreen dye-, OliGreen dye- and RiboGreen dye-based fluorescence assays are up to 10,000-fold more sensitive than UV absorbance measurements and at least 400-fold more sensitive than assays that use the Hoechst 33258 dye requiring much less sample for quantitation.

Accuracy

Unlike measurements of UV absorbance, these assays are not affected by the presence of proteins, free nucleotides or very short oligonucleotides. This makes quantitation of intact oligonucleotides and nucleic acids much more accurate in complex mixtures such as serum or whole blood.

Precision

The average coefficients of variance of triplicate assays using these reagents are typically less than 1.5%.

Simplicity

These assays have a very simple protocol that requires no separation steps making them ideal for automated, high-throughput measurements.

Broad dynamic range

Quantitation is accurate over four orders of magnitude for the PicoGreen and OliGreen assays, with a single dye concentration. The RiboGreen assay is accurate over three orders of magnitude.

Instrument compatibility

Quantitation assays can be performed using a fluorescence microplate reader with standard fluorescein filters, a relatively inexpensive filter-based spectrofluorometer or a standard spectrofluorometer.

1.5.3.2 Other Nucleic Acid Binding Dyes

Within this project, Ethidium bromide has been used to visualise DNA within agarose gel matrices thus enabling the determination of nucleic acid content. As explained in Section 1.4, EtBr is an intercalating dye, which at saturation sees the one molecule of EtBr in every 4 -5 base pairs in a dsDNA molecule, so complexes are easily visualised. Excitation maximum is at 500 nm but there is also absorption of UV light at 254, 302 and 366 nm. Fluorescent emission is at 590 nm. Illumination is generally done at 302 nm which represents a compromise between reduced quantum yield at 366 nm and increased nicking at 254 nm which can be a problem if DNA bands are to be excised and purified (Maniatis et al., 1989).

DNA stains based on unsymmetrical cyanine dyes have gained in popularity in recent years. SYBR Green I and II, used briefly on agarose gel matrices in this project, fall into this family of dyes. The SYBR Green dyes are more sensitive than EtBr with lower mutagenicity (Singer et al., 1999). SYBR Green I can detect as little

as 60 pg of dsDNA and the ssDNA binding SYBR Green II can bind as little as 100 pg of RNA. The fluorescent enhancement of the SYBR Green dyes, when bound to nucleic acids, are also more than 10 times the enhancement of EtBr complexes and thus there are lower background signals. Excitation and emission wavelengths are 494 and 521 nm respectively (Singer et al., 1999).

SYBR Gold is another ultra sensitive stain available for detecting DNA and RNA. It is used in gels utilising a standard 300 nm UV transilluminator and Polaroid 667 black-and-white print film. The SYBR Gold stain represents a further improvement over SYBR Green I and SYBR Green II gel stains for gel analysis as the sensitivity is greater , >1000-fold fluorescence enhancement and a quantum yield of ~0.6 (Molecular Probes, manufacturer's data, www.probes.com). The stain also penetrates gels rapidly and stains thick and high-percentage gels better than other post-electrophoresis stains. The SYBR Gold stain may be used for a variety of gel types, including high-percentage agarose, glyoxal/agarose, formaldehyde/agarose, native polyacrylamide- and urea-polyacrylamide gels. No wash step is required in order to achieve maximal sensitivities. It is also easy to use, economical and compatible with other molecular biology techniques. The stain does not interfere with restriction endonuclease or ligase activity or with subsequent PCR reaction, it is also compatible with both Northern and Southern blotting. The SYBR Gold stain is also easily removed from dsDNA by simple ethanol precipitation, leaving templates ready for subsequent manipulation or analysis.

Table 1.6 provides a comparison of the nucleic acid stains in current use and their relevant properties.

Nucleic Acid Stain	Excitation Wavelength	Emission Wavelength	Qualitative Sensitivity	Advantages/ Disadvantages
Ethidium Bromide	254, 302, 366, 500 nm	590 nm	Relatively insensitive	Economical and well known
PicoGreen	488, 502 nm	523 nm	Can accurately quantitate as little as 25 pg/mL of dsDNA in a fluorometer. The PicoGreen assay is more than 10,000 times as sensitive as conventional UV absorbance measurements at 260 nm (an A260 of 0.1 corresponds to an ~5 µg/mL dsDNA solution) and at least 400 times more sensitive than the Hoechst 33258 dye-based assay.	Can be used in solution. Higher sensitivity means that lower volumes can be used.
SYBR Green	494 nm	521 nm	SYBR Green I can detect as little as 60 pg of dsDNA and the ssDNA binding SYBR Green II can bind as little as 100 pg of RNA. At least an order of magnitude greater than that of ethidium bromide when detected by photography and low background fluorescence	Used in agarose gels. Significantly less mutagenic than EtBr by Ames testing.
SYBR Gold	300, 495 nm	537 nm	Greater than 10-fold more sensitive than ethidium bromide for detecting DNA and RNA in gels. SYBR Gold stain is 25–100 times more sensitive than ethidium bromide	Used in agarose gels
Hoescht dyes	350 nm	461 nm	Used mainly for the staining of whole living cells and thus cannot be compared to EtBr or other dyes above.	The Hoechst 33258 and Hoechst 33342 dyes have complex, pH-dependent spectra when not bound to nucleic acids, with a much higher fluorescence quantum yield at pH 5 than at pH 8. Thus, not suitable for a assay utilising shifts in pH.

Table 1.6: Properties of commonly used Nucleic Acid Stains

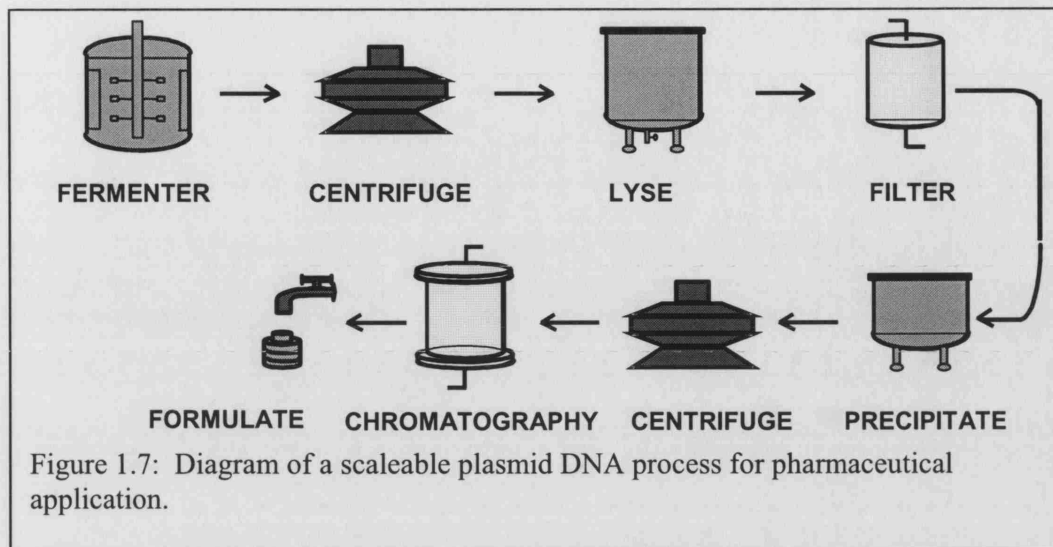
1.6 Thesis Outline

This thesis sees the characterisation of plasmid streams at different stages across a potentially scaleable process, as shown in Figure 1.7. Chapter 2 describes the methods and protocols used within this project. In Chapter 3, the cell growth kinetics and plasmid yield of a 70 L and 450 L fermentation are reported. In Chapter 4, the effect of large-scale cell harvest by continuous centrifugation on the plasmid yield and chromosomal DNA contamination is investigated, along with the analysis of lysis and lysate clarification methods. In Chapter 5, a microplate based assay is developed for use on purified plasmid DNA and bacterial artificial chromosomes. Chapter 6 investigates the application of this microplate based assay to impure cell process streams and in Chapter 7 the microplate based assay is adapted for industrial use with a robotic liquid handling system.

Thus, this thesis considers the critical challenges presented at key unit operation stages. It investigates the use of methods that could potentially be used to gain a better understanding of the effect of process parameters and the engineering environment on the plasmid product and its process contaminants.

The final chapter summarises the project and considers the direction of current assay development and validation of biochemical and pharmaceutical product, (Chapter 8). The major issues presented by this project will be considered as well as the steps necessary to carry on the most interesting aspects and their application to the modern pharmaceutical industry.

Papers submitted and published as part of the research are shown in Appendices 1 and 2. Appendix 3 shows detailed protocols used for the production of high quality plasmid DNA using Qiagen kits.



2 METHODS AND MATERIALS

2.1 Introduction

The methods covered in this section were used over the three years of the project. They were, for the most part, standard microbiology techniques for the inoculation and growth of *E. coli* cultures, the subsequent harvest of cells and the extraction and purification of plasmid DNA, as discussed in Maniatis et al., (1989) and Ausubel et al., (2002). Techniques used to characterise this DNA product as well as the new assays developed and the methods, such as protein analysis, used to validate these procedures are also described in this section. All raw materials such as chemicals and media components were provided by Sigma-Aldrich (Poole, UK) unless otherwise stated.

2.2 Broth and Buffer Recipes

2.2.1 Fermentation Broths

Luria Bertani Broth (LB):

10 g Bacto- tryptone, 10 g NaCl, 5 g yeast extract.

Made up to 1L with reverse osmosis (RO) water, autoclaved and allowed to cool before use.

Superbroth:

32 g Bacto-tryptone, 20 g yeast extract, 5 g NaCl.

Dissolved solids in 800 mL of RO water, added Sodium hydroxide (NaOH) until pH 7.4, made up to 1L with RO water, autoclaved and allowed to cool before use.

Terrific Broth:

12 g Bacto- tryptone, 24 g yeast extract, 4 mL glycerol.

Dissolved in 900 mL RO water, autoclaved and cooled to 60 °C or less, added 100 mL of sterile solution of 0.17M KH_2PO_4 , 0.72 K_2HPO_4 . In order to make 100 mL of salt solution, dissolved 2.31 g KH_2PO_4 and 12.54 g K_2HPO_4 in 90 mL RO water, adjusted volume to 100 mL, autoclaved and cooled.

2.2.2 Stock Buffer Recipes

1 M Tris- Cl:

For 1 L, used 121.1 g Trizma base (Trishydroxymethylmethamine), 800 mL RO water, 42 mL concentrated HCl. Cooled to room temperature, adjusted volume to pH 8, and made volume up to 1L and autoclaved. Buffer was stored at room temperature.

0.5 M EDTA:

For 1 L, used 186.1 g $\text{EDTA} \cdot 2\text{H}_2\text{O}$ (ethylenediaminetetraacetic acid), 800 mL RO water, 20 g NaOH pellets. Stirred vigorously and allowed mixture to cool. Adjusted pH of buffer to pH 8 and autoclaved. Buffer was stored at room temperature.

10 % SDS:

For 1 L, used 100 g Sodium dodecyl sulphate (SDS), 900 mL RO water, heated mixture to 68 °C to dissolve SDS, adjusted volume to 1L and stored at room temperature. This solution was not autoclaved.

10 M NaOH:

Dissolved 40 g NaOH pellets in RO water, allowed to cool and made up to 100 mL.

5 M Potassium Acetate:

For 1L, dissolved 491 g potassium acetate in RO water. Autoclaved and cooled.

3 M Sodium acetate:

Added 408.1g Sodium acetate to 800 mL RO water, adjusted pH to 5.2 with glacial acetic acid, adjusted volume to 1 L and autoclaved.

2.2.3 Buffer Recipes and preparatory methods for DNA Extraction

TE buffer pH8- Solution P1 (used to resuspend cells):

10 mL 1M Tris- Cl solution (pH 8), 2 mL 0.5M EDTA solution (pH 8), adjusted volume to 1 L, autoclaved. Treated bovine pancreatic RNase A to a working concentration of $100 \mu\text{g mL}^{-1}$ was added, if necessary, just before use.

Treatment for Bovine Pancreatic RNase:

This treatment ensured that RNase A was free of contaminating DNase that could destroy the plasmid product. RNase A was dissolved to a concentration of 110 mg mL^{-1} in 0.01 M sodium acetate pH 5.2 using sterile ultrapure water. The mixture was heated to 100°C for 15 minutes in a water bath and allowed to cool slowly to room temperature. The pH was adjusted by adding 0.1 volumes of 1 M Tris-Cl and the mixture was dispensed in aliquots and stored at -20°C . $1 \mu\text{L}$ of RNase was used for every mL of bacterial cell resuspension.

Lysis buffer (0.2 M NaOH, 1 % SDS buffer)- Solution P2:

This solution was always freshly made and was never autoclaved. For 1 L of solution, 20 mL of 10 M NaOH and 100 mL of 10% SDS were used, the buffer volume was adjusted to 1L with sterile ultrapure water.

Neutralisation buffer (3 M potassium acetate)- Solution P3:

For 1 L of buffer, 115 mL glacial acetic acid, 600 mL 5M potassium acetate stock, and 285 mL RO water were mixed. The pH was adjusted to 5.5 with glacial acetic acid and adjusted volume to 1 L with water. The buffer was autoclaved and cooled.

2.2.4 Recipes and preparatory methods for agarose gel electrophoresis

5X Tris Borate Buffer (TBE) Buffer:

Tris Base (Tris hydroxymethylmethamine, $M_r=121.13$)	54.43 g
Boric Acid	27.78 g
EDTA	1.85 g

Dissolved solids in 800 mL of ultrapure water and adjusted volume to 1L. Did not autoclave.

For gels (both 1D electrophoresis and pulsed field) 0.5X TBE was used, which was made by diluting 5X stock buffer to the required strength with ultrapure water. The diluted solution was always freshly made.

Preparation of Agarose Gels:

To make a gel the following steps were followed:

- The open edges of an electrophoresis tray were sealed with autoclave tape or Perspex barrier to form a mould.
- The requisite amount of powdered agarose was weighed to make a gel of 130 mL volume, for a 0.8 % gel, 1.04 g of agarose was used, for a 1% gel , 1.3 g of agarose was used.
- The powder was placed in a clean Erlenmeyer flask and 130 mL of 0.5X TBE buffer was added.
- The mixture was heated in the microwave for 1 minute and swirled gently. The mixture was then heated again for a further minute.
- The solution was cooled to 60 °C. Using a 1 mL Gilson pipette, the edges of the mould were sealed with a small quantity of the agarose solution. The combs were then positioned in the mould.

- The agarose solution was then transferred to an Erlenmeyer labelled “ethidium bromide”.
- 5 μL of ethidium bromide stock solution was added; this gave a final concentration of $0.05 \mu\text{g mL}^{-1}$.
- The mixture was swirled gently.
- The warm agarose solution was then poured into the mould. If air bubbles formed, they were pricked to leave the gel matrix free of trapped air.
- The gel was set for 45 minutes at room temperature and the comb was then carefully removed.
- The gel matrix was wrapped in cling film, placed in a flat bottomed container and stored at 4°C until further use.

2.3 Description of Bacterial strains used

Escherichia coli (*E. coli*) was used in order to propagate the plasmids studied within this thesis. The bacterial map of *E. coli* is shown in Figure 2.1. The strains used were DH5 α and DH10 β .

DH5 α is a well-known and versatile bacterial strain used in many everyday cloning applications. It supports the blue/white screening of recombinant clones due to lacZM15. The *recA1* and *endA1* mutations in DH5 α increase insert stability and improve the yield and quality of plasmid DNA prepared from minipreps.

Genotype:

F⁻ ϕ 80dlacZ Δ M15 Δ (lacZYA-argF) U169 *recA1 endA1 hsdR17*(r_k⁻, m_k⁺) *phoA supE44 λ ⁻ thi-1 gyrA96 relA1*

2.4 Description of Plasmids used

The plasmids used within this project are as follows: 6.9kb pSV- β - Galactosidase (Promega Corp., Madison, WI, USA), the 20 kb plasmid pQR150 (Jackson et al., 1995), the BAC vectors used in this project were 116, 172 and 242 kb containing inserts of 104, 160 and 230 kb, respectively. A summary is shown in Table 2.1.

Plasmid	Size
pSV β	6.9 kb
pQR150	20 kb
p5176	116 kb
p5206	172 kb
p5204	242 kb

Table 2.1: Summary of plasmids used in the thesis

The BAC vectors all exist as one copy per bacterial cell as their origin of replication is based on the F1 plasmid. The vectors also contain the family of repeats from the Epstein Barr virus latent origin of replication which allow greater episomal stability (Wade-Martins et al., 1999). These BACs contains an insert for chloramphenicol resistance. The map is shown in Figure 2.2A.

pSV- β -Galactosidase is a pUC based plasmid with high copy number (~ 500 copies per cell). The pSV- β -Galactosidase vector is a modification of pRSV- β -Gal with SV40 and pUC18 sequences substituted for RSV and pBR322 sequences. The pSV- β -Galactosidase Vector will express β -galactosidase in *E. coli* due to the presence of the *E. coli* gpt promoter located upstream of the lacZ gene. Colonies of *E. coli* containing the pSV- β -Galactosidase Vector appear blue when plated on media containing X-Gal (from Promega Corp., manufacturer's data, www.promega.com). The plasmid contains an insert for ampicillin resistance as shown in the map in Figure 2.2B.

pQR150 is a 20 kb derivative of pBGS 18 (Jackson et al., 1995) and also exists in at high copy number in the bacterial cell; the plasmid contains an insert for kanamycin resistance. Genes for the benzoate meta-cleavage pathway of *Pseudomonas putida*, which encode catechol-2,3-dioxygenase activity, were cloned into pBGS18 (Km^r) by J. M. Ward, and designated pQR150, the plasmid map is shown in Figure 2.2C.

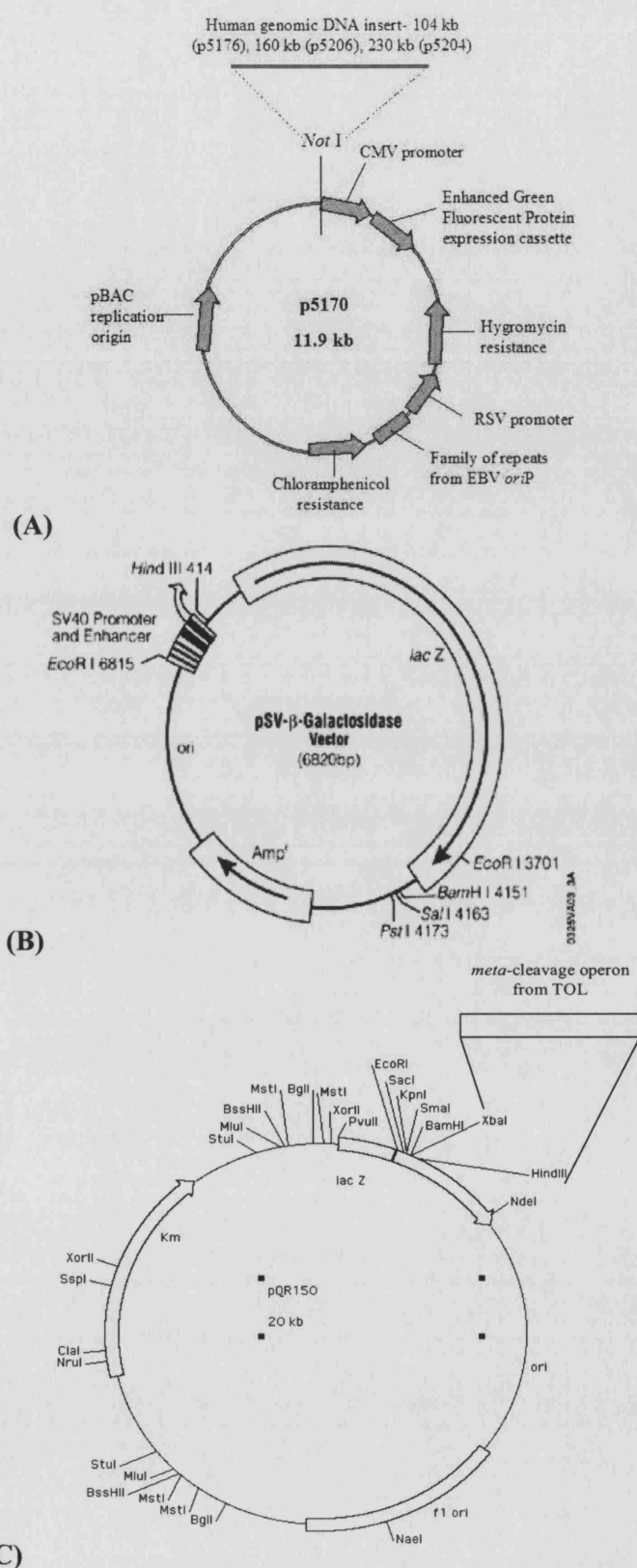


Figure 2.2: (A) Diagram of cloning scheme of p5176, p5204 and p5206 (Wade-Martins et al., 1999); (B) Plasmid map of pSVβ (Promega Corp. Madison, WI, USA. www.promega.com); (C) Plasmid map of PQR150 (provided by JM Ward).

2.5 Experimental Methods

2.5.1 Source of Bacterial Strains and Plasmids

Plasmid pSV β (Promega Corp., Madison, WI, USA), a 6.9 kb and pQR150 (Jackson et al., 1995), a 20 kb plasmid, were transformed and propagated in *E. coli* DH5 α (Gibco-Life Technologies, Gaithersburg, MD, USA). Bacterial artificial chromosomes (BAC) based episomal shuttle vectors p5176, p5204 and p5206, which are 116, 172, and 242 kb in size respectively as described above (Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, UK). BACs were transformed and propagated in *E. coli* DH10 β (Wade- Martins, 1999). The host bacterial strains were maintained in 20% (v/v) glycerol at -70 °C.

2.5.2 Fermentation

2.5.2.1 Shake Flask Cultures

Culture, harvesting and storage conditions were as described in Levy et al, 2000. Cultures of *E. coli* DH5 α pSV β and DH5 α pQR150 contained ampicillin, 100 $\mu\text{g mL}^{-1}$, kanamycin, 50 $\mu\text{g mL}^{-1}$ respectively. DH10 β was grown with chloramphenicol; 12.5 $\mu\text{g mL}^{-1}$ on plates and 5 $\mu\text{g mL}^{-1}$ in shake flask cultures. Selective plates and nutrient media used for the preparation of p5176 were freshly prepared with overnight incubation in light of the low concentration of antibiotic. Petri dishes of DH5 α bacteria containing either no plasmid or plasmids pSV β and pQR150 were incubated at 37 °C overnight after inoculation from glycerol stocks stored at -70 °C.

The standard fermentation method started with the use of an overnight fermentation with shaking (using an orbital shaker at 200 rpm) of *E. coli* with appropriate antibiotic, inoculated with a single colony of bacteria from the selective

Petri plate. This fermentation is used in a 1/250 or 1/500 dilution to inoculate the main secondary shake flask fermentation (working volume 500 mL), which is incubated at 37 °C for 8 hours. When there were time constraints on the fermentation, it was modified so that the primary fermentation was 8 hours in length and the main fermentation was inoculated at low dilution (usually 1/100) and incubated overnight at 37 °C with shaking for no more than 16 hours. Rich media were used for the fermentations: Terrific, Luria Bertani and Superbroth as described in section 2.2.1.

2.5.2.2 Large- scale Cultures

Plasmid pQR150 (20kb), was transformed and propagated in *E. coli* DH5 α , glycerol stocks were stored at -70 °C. The media used was Terrific broth (12 g L⁻¹ Bacto- tryptone, 24 g L⁻¹ yeast extract, 4 mL L⁻¹ glycerol, 0.017 M KH₂PO₄, 0.072 M K₂HPO₄) and cultures of DH5 α pQR150 contained kanamycin, 50 μ g mL⁻¹. Overnight, 2 L shake flask cultures (working volume 500 mL) were used to inoculate a Series 2000 LH 75 L bioreactor (Inceltech, Pangbourne, UK) at a starting optical density at 600 nm (OD₆₀₀) of 0.5 and working volume of 45 L. The antifoam, polypropyleneglycol (PPG), was added at a concentration of 0.36 mL L⁻¹. Bioreactor conditions were 37 °C, 500 rpm agitation, 22.5 Lmin⁻¹ airflow rate. The pH was maintained at 7 and control was achieved through the addition of 1M sulphuric acid and 1M NaOH when necessary. After 13 hours of growth the culture was used to inoculate 300 L of Terrific broth to a starting OD₆₀₀ of 0.5. The culture was grown within a 450 L capacity Chemap bioreactor (Chemap AG, Maenndorf, Switzerland) for 11 hours to a final OD₆₀₀ of 20.35. Initial bioreactor conditions were 37 °C, 200 rpm agitation, 180 L min⁻¹ airflow rate, the speed and airflow increased over the course of the fermentation to 400 rpm and 250 L min⁻¹ airflow in an attempt to raise

the dissolved oxygen tension (DOT). Foaming and pH were controlled as for the 75 L fermentation. At the end of the fermentation the culture was crash cooled to 10 °C and harvested immediately. Samples were taken every hour during the fermentation, optical density at 600 nm was measured, and readings of dissolved oxygen tension, agitation and airflow rate were also recorded. Fermentation samples were micro centrifuged at 13000 rpm to harvest cells and the resulting cell paste and supernatant were stored at -20 °C.

2.5.3 Harvest of bacterial cultures

2.5.3.1 Small-scale fermentation harvest

Small-scale shake flask fermentations were usually harvested using the Beckman (Beckman Instruments, Palo Alto, CA, USA) J2-MI batch centrifuge, using the JA-10 rotor (inner radius 0.04 m, outer radius 0.075 m) at 6000 rpm. Harvested cell paste and sludge were stored at -20 °C. Samples of less than 2 mL in volume were harvested by micro centrifuge at 13000 rpm and the product stored at -20 °C.

2.5.3.2 Large- scale fermentation harvest

The 300 L culture was harvested using a number of methods. The rationale for operating conditions chosen for the pilot-scale centrifuges has been previously described (Boychyn et al., 2000; Boychyn et al., 2001). The CSA-1 (Westphalia Separator, Oelde, Germany) disk-stack centrifuge, outer disc radius 0.053 m, inner disc radius 0.021 m, 43 disks per stack, was operated at flowrates 50 L h⁻¹ and 250 L h⁻¹. A diagram of this centrifuge is shown in Figure 2.3. The CARR (Carr Separations, Franklin, MA, USA) P6 powerfuge tubular bowl centrifuge, inner weir radius 0.076 m, inner bowl radius 0.051 m, inner length of bowl 0.127 m was

operated at flowrates 30 L h^{-1} and 60 L h^{-1} . The Beckman (Beckman Instruments, Palo Alto, CA, USA) J2-MI laboratory scale batch centrifuge, using the JA-10 rotor (inner radius 0.04 m, outer radius 0.075 m) was operated at 6000 rpm. Harvested cells were stored at $-20 \text{ }^{\circ}\text{C}$.

Centrifugation may be described as the separation of particles or fluids of varying densities by exposure to a centrifugal force due to the spinning of the centrifuge assembly. As the particles are exposed to the force the heavier particles move outwards where they may be separated either by decanting the lighter portion at the end of the centrifugation and leaving a cell pellet or by the continuous discharge of the heavier element during centrifugation. In fermentation broth, the cells represent the heaviest element and centrifugation represents a time efficient method of large scale clarification for process streams. In the disk stack centrifuge, the rotating disks are loaded with the broth input from the top, the cells within the broth experience a centrifugal force due to the spinning disks and move towards the lower region of the assembly as shown in Figure 2.3. The solids are discharged intermittently from this area through a tube at the base of the centrifuge assembly. The CARR P6 is a solid titanium bowl centrifuge, with rotating bowl and full length scraper assembly for rapid, contamination-free, solid harvesting. The unit also has water cooling capability which was used in the pilot plant run. The CARR centrifuge also intermittently discharges solids.

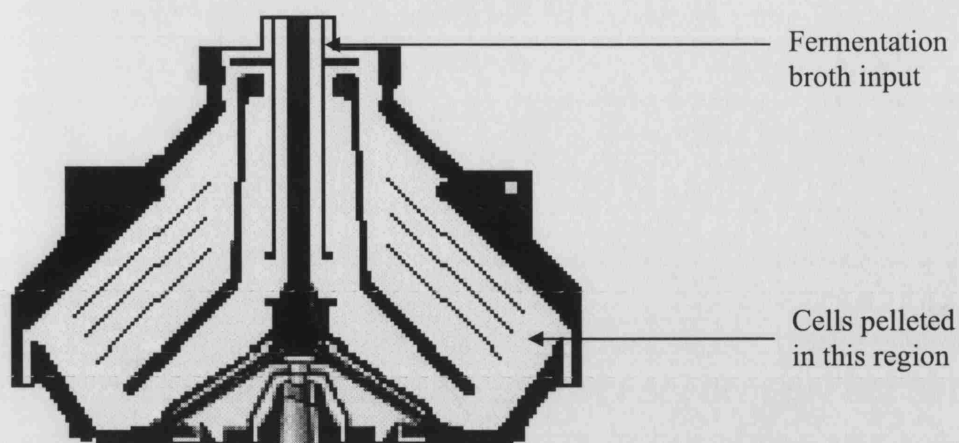


Figure 2.3: A diagram of a continuous disk stack centrifuge, solid particles are removed in flow between closely stacked cones. Clarified effluent is withdrawn from the top of the unit, solids are discharged from the bottom, (adapted from Bailey and Ollis, 1986, Biochemical Engineering Fundamentals).

2.5.4 Plasmid Extraction and Purification

2.5.4.1 Alkaline Lysis

2.5.4.1.1 Alkaline lysis of small scale fermentation

Cell paste harvested from shake flask fermentation was resuspended with TE buffer or Qiagen buffer P1 (one fifth of the volume before cell harvest- i.e. resuspension in 100 mL of cells obtained from 500 mL fermentation). The resuspension buffer contains 100 $\mu\text{g mL}^{-1}$ treated RNase A. The resuspension was mixed with an equal volume of lysis buffer or Qiagen buffer P2 (Qiagen Ltd, West Sussex, UK). The reaction was not allowed to continue for more than 5 minutes before the addition of an equal volume of neutralisation buffer or 1.3 volumes of Qiagen buffer P3. The mixture was kept on ice for 5 minutes and then centrifuged in order to isolate the precipitated cell debris and chromosomal DNA. Centrifugation

was carried out in either a microcentrifuge (at 13000 rpm) or using the Beckman batch centrifuge using the JA-10 (10000 rpm) or JA-17 (17000rpm) rotors. The low pH of the Potassium acetate solution effectively neutralizes the NaOH and when the pH of the solution returns to near-neutrality then the macromolecules renature. The proteins and large DNA molecules do not renature correctly as they form hydrophobic, ionic and hydrogen bonds with each other non-specifically because the correct conformation of the molecule was not maintained during denaturation. The plasmid DNA molecules, however, do not fully denature because of their small circular nature. Although the hydrogen bonds between base pairs were broken by the high pH, they reform correctly when the pH is lowered. The large DNA molecules (chromosomal DNA) and proteins form precipitates because they bind to each other in a large aggregate but the plasmids don't precipitate because they renature correctly and don't become part of the large multi-molecule aggregates. Thus plasmid DNA remains in solution while most of the protein and other DNA molecules precipitate.

2.5.4.1.2 Alkaline lysis of cell paste from large-scale fermentation

Cell pastes were resuspended with gentle shaking in TE buffer (50 mM Tris-Cl, 100 mM EDTA, pH 8.0) at $OD_{600} = 50$ or 65 depending on the experiment. Volumes of this suspension were taken for lysis at small-scale (0.5 mL), and medium-scale (300 mL). Cells were lysed using a modified alkaline lysis technique as previously described (Levy et al., 1999). At the 300 mL scale, resuspension volume was 100 mL of TE buffer, 100 mL of lysis buffer was added (200 mM NaOH, 1% SDS w/v), gently mixed and left for ≤ 10 minutes followed by the addition of 100 mL of neutralisation buffer (3.0 M Potassium acetate, pH 5.5). Additionally, some

small-scale samples were held at the lysis stage with NaOH and SDS for 1 hour before proceeding with neutralisation, centrifugation and DNA precipitation. This was done to model pockets of cell suspension overexposed to lysis buffer as may occur due to inefficient mixing at large-scale. After neutralisation, flocculated cell debris, proteins and chromosomal DNA were either centrifuged using the Beckman centrifuge (JA-10 rotor) or filtered using Whatman no. 1 filter paper, pore size 11 μm (Whatman International Ltd, Maidstone, UK). DNA from samples of clarified lysate was then precipitated using the isopropanol precipitation technique (IPA), washed with ethanol, resuspended in TE buffer containing 100 $\mu\text{g mL}^{-1}$ RNase A and incubated for 1 h at 37 °C to remove contaminating RNA. Volumes of cell suspensions were also taken at the small-scale and microcentrifuged using the Hereaus Biofuge (Fisher Scientific, Loughborough, UK). The supernatant was decanted and isopropanol precipitated with addition of NaCl (0.2 M final concentration); the precipitated DNA was then washed with ethanol and treated to remove contaminating RNA where required.

2.5.4.2 Lysozyme and Triton

Samples of cell paste obtained from small-scale fermentation were resuspended and mixed with an equal volume of lysis solution containing 0.2% Triton and 10 mg mL^{-1} lysozyme (Ausubel et al., 2002). The mixture was gently shaken and incubated at room temperature for 10 minutes. Samples were diluted with TE buffer before analysis using PicoGreen.

2.5.5 Isopropanol Precipitation

2.5.5.1 Precipitation of small scale samples

Samples of clarified lysate were mixed with 0.7 volumes of isopropanol and centrifuged, then washed with an equal volume of 70% ethanol in ultrapure water and re-centrifuged. Centrifugation was carried out using either the Hereaus Biofuge at 13000 rpm or the Beckman batch centrifuge with JA-10 (10000 rpm) or JA-17 (17000 rpm) rotor. After ethanol washing the precipitate was drained and dried for ten minutes before resuspension with TE buffer. Samples were then either used straight away or stored at -20°C .

2.5.5.2 Precipitation of samples derived from large scale pilot plant fermentation

DNA from samples of clarified lysate was precipitated using 0.7 volumes isopropanol centrifuged using the Hereaus Biofuge at 13000 rpm. The isopropanol was discarded and the precipitate washed with 1 volume 70 % ethanol and re-centrifuged at 13000 rpm. After discarding the ethanol, the precipitated DNA was resuspended in TE buffer containing $100\text{ }\mu\text{g mL}^{-1}$ RNase A and incubated for 1 hr at 37°C to remove contaminating RNA.

Volumes of cell suspensions were also taken at small scale and microcentrifuged using the Hereaus Biofuge (Fisher Scientific, Loughborough, Leics, UK). The supernatant was decanted and 0.7 volume of isopropanol added with NaCl (0.2 M final concentration); the precipitated DNA was then washed with ethanol and treated to remove contaminating RNA.

2.5.6 Chromatographic purification: Qiagen

DNA from clarified lysate derived from the small scale fermentations of *E. coli* DH5 α pSV β , pQR150, no plasmid and DH10 β p5176, p5204 and p5206 were purified using either Qiaprep Spin Miniprep, Mini or Maxi prep kits and their respective purification protocols for high and low copy number vectors (Qiagen Ltd, West Sussex, UK). The full protocols for Qiagen Spin Miniprep, Mini and Maxi prep kits are given in Appendix 3. Separation is achieved through anion exchange chromatography. DNA concentrations were determined by UV spectrophotometry at 260 nm. For samples obtained from the large-scale fermentation, both clarified lysates and isopropanol precipitates of pQR150 DNA and DNA extracted from the host cell *E. coli* DH5 α (no plasmid) were purified using Qiagen Miniprep kits and purification protocols. DNA concentrations were determined by UV spectrophotometry at 260 nm.

2.5.7 Determination of plasmid yield by UV Spectrophotometry

This method was used for the accurate quantitation of DNA. The presence of contaminants such as salts, proteins, RNA and single stranded DNA give a significant increase in the signal so it is not a very robust method and can only be used for very pure samples (i.e. Qiagen purified DNA, and not isopropanol precipitated samples). Both RNA and DNA absorb UV light thus making it possible to detect and quantify either nucleic acid at concentrations as low as 2.5 $\mu\text{g mL}^{-1}$. The nitrogenous bases in nucleotides have an absorption maximum at about 260 nm. The absorbance at 260 nm of a 50 $\mu\text{g mL}^{-1}$ solution of double stranded DNA is equal to 1. The following calculation was used to quantify DNA concentration in samples:

$$\text{DNA concentration } (\mu\text{g mL}^{-1}) = (A_{260}) \times (\text{dilution factor}) \times (50 \mu\text{g DNA mL}^{-1}) / (1 A_{260} \text{ unit})$$

Quartz cuvettes were used to obtain the absorbance measurements and the machine used was the Biomate 3 Spectrophotometer (Thermo Spectronic, Madison, WI, USA), making dilution where appropriate so that readings were taken between 0.1 and 1.0. In contrast to nucleic acids, proteins absorb maximally at a wavelength of 280 nm, due mostly to tryptophan residues. The absorbance of a DNA sample at 280 nm gives an estimate of the protein contamination of the sample. The ratio of the absorbance at 260 nm/ absorbance at 280 nm is a measure of the purity of a DNA sample; it should normally be between 1.6 and 1.8, and for highly pure samples the ratio should be between 1.7 and 1.8. A ratio of less than 1.6 indicates high levels of protein contamination in the sample. Samples of pure DNA were also scanned using the spectrophotometer between wavelengths of 220 and 320nm.

2.5.8 Degradation of cell paste and plasmid

2.5.8.1 Chemical and shear degradation of pure plasmid solution

Pure preparations of pSV β , pQR150 and p5176 were diluted to concentrations ranging from 100 to 500 ng mL⁻¹ in TE buffer and incubated in a water bath at 60 °C to accelerate the generation of DNA strand breaks through chemical degradation (Evans et al., 1999). The solutions (hereafter referred to as chemically degraded samples) were periodically sampled and aliquots kept at -20°C. Samples of 10 mL volume of pSV β and p5176 at a concentration of 11 ng mL⁻¹ in TE buffer were subjected to average shear rates between 1.6×10^4 s⁻¹ and 3.3×10^5 s⁻¹ utilising an electric small scale shear device consisting of a Perspex chamber of 20 mL capacity and thin steel disc. The chamber was completely filled with solution and the disc rotated at high speed. Levy et al., 1999 and Levy et al., 2000, describe the machine

and methods used. A half full chamber was used to inflict the highest shear possible as theoretically shear is worst where there is a significant the gas/ liquid interface.

2.5.8.2 Damage of bacterial suspensions – by temperature and shear

Bacterial cell pastes from large-scale fermentation of DH5 α pQR150 (harvested using tubular bowl centrifuge) were resuspended to OD_{600nm} = 50 and incubated at temperatures of 4, 13, 28 and 37 °C with and without shaking (orbital shaker 200 rpm) for 2, 6 and 16 hours. At the higher temperatures nucleic acids are degraded by nucleases as seen in Chapter 4.

Bacterial cell suspensions were subjected to average shear rates between $1.6 \times 10^4 \text{ s}^{-1}$ and $3.3 \times 10^5 \text{ s}^{-1}$ using the methods described in section 2.5.8.1 and by Levy et al., 1999 and Levy et al., 2000.

2.5.9 Agarose Gel Electrophoresis

2.5.9.1 Introduction

The term ‘electrophoresis’ refers simply to the movement of particles by an electric force. This method uses current to achieve separation based on mass-charge ratio and size. Nucleic acids are negatively charged due to their ionised phosphate groups. As the size of nucleic acids increases, charge also increases so the mass-charge ratio remains constant. Thus electrophoretic separation of these compounds is almost entirely based on size. This is achieved with the sieving effect of the gel matrix. It is not very accurate method for quantitation; error is 10 - 20% and a calibration curve with known amounts of plasmid DNA should be loaded in each gel. However, unlike spectrophotometry, rather impure samples can be used, i.e. isopropanol precipitated DNA resuspended in TE. Various dyes can be used to detect

the DNA: in this work ethidium bromide was used. Ethidium bromide is an intercalating dye that binds to dsDNA, ssDNA and RNA. Runs can take from 1 to 14 hours but up to 40 samples can be electrophoresed in the same gel. It is useful to visualize different conformations of the plasmid and presence of contaminant nucleic acids such as chromosomal DNA or RNA.

Current laboratory techniques are performed in a gel matrix (in this case, agarose, a seaweed extract) perfused by an aqueous solution of buffer salts. A homogeneous buffer system was used, where the identity and concentration of buffer components are the same in the gel and the tanks. The buffer performs the functions of protecting the samples by cooling and most importantly, carrying the current. The buffer used was Tris- borate (TBE) which is popular in molecular biology as both the Tris base and borate are uncharged part of the time at the desired pH, which reduces their electrophoretic mobility. This allows excellent buffering capacity and sample stability without depletion of ions during long runs or unacceptably high conductivity. Agarose gel electrophoresis is a major part of the project, it is used to verify DNA isoforms, and as a standard method for the comparison and eventual correlation with results from the assays that have been developed.

2.5.9.2 Agarose gel electrophoresis in one dimension

The preparation of gels and electrophoresis running buffers was described previously. As sample separation depends on DNA size the running conditions will vary from plasmid to plasmid. Plasmids pQR150 and pSV β were analysed using the standard one-dimensional method. Samples were loaded as detailed below:

Loading of samples in the gel:

- 5- 20 μ L of Sigma gel loading buffer were pipetted into eppendorfs for each sample.
- 5- 20 μ L of λ HindIII marker was pipetted into its eppendorf and 5- 20 μ L of all other samples were placed in their eppendorfs. Larger quantities of sample were used when DNA quantities of unknown samples were expected to be low, in these situations low marker volume was used.
- The eppendorfs were placed in the Haearus bench micro centrifuge and spun at 13000 rpm for 30 seconds.
- The gel was removed from the mould and slid into the gel box; it was ensured that the wells were in the correct position, so that the DNA ran through the gel towards the anode.
- 0.5X TBE buffer was poured into the gel box at both ends until the buffer just covered the top of the gel.
- The contents of each eppendorf were pipetted into the wells of the agarose gel, with care taken not to spill the samples or push air into the wells.
- The gel box was covered and the power pack controls set to 80V, for 2 hours for pSV β and 40V for 2- 14 hours for pQR150 samples. The samples were then electrophoresed.

Analysis of Agarose Gels:

After electrophoresis the gels were taken out and DNA visualised with transillumination by UV light using the UVP 5000 gel documentation system (Ultra Violet Products Ltd, Cambridge, UK) or the Bio-Rad gel documentation system (Bio-Rad, Hercules, CA, USA). The supercoiled (SC), open circular (OC) and linear DNA bands were analysed via densitometric scanning and SC DNA values were multiplied

by a correction factor of 1.36 to account for the differential binding of ethidium bromide (Projan et al., 1983).

2.5.9.3 Pulsed Field Gel Electrophoresis

Pulsed field gel electrophoresis uses the periodic changes in the angle of the electric field to resolve DNA molecules up to several million bases in length. For the experiments used in this thesis, a 1 % agarose gel was slid onto the electrophoresis platform of the Chef-DR[®] II pulsed-field electrophoresis system (Bio-Rad, Hercules, CA, USA) and 0.5X TBE buffer poured into the gel box to cover the gel to a depth of 1-2 mm. In total the apparatus requires almost 2 L of buffer. Recirculation was adjusted to $> 100 \text{ mL min}^{-1}$ and the cooling switched on to a set point of 13 °C. The system was allowed to equilibrate and chill to the temperature set point. The bacterial artificial chromosome (BAC) samples were loaded onto the gel as described previously and electrophoresed in TBE buffer at 6V for 22.5 hours on the Chef-DR[®] II pulsed-field electrophoresis system (Bio-Rad, Hercules, CA, USA), initial switch time 0.8s and final switch time 21.1s. The gels were stained after electrophoresis by placing the gel in a box containing 0.5 L of 0.5X TBE buffer containing $0.05 \text{ } \mu\text{g mL}^{-1}$ of ethidium bromide. The gel was gently shaken on a rocking platform for 30 minutes and then destained using RO water. DNA was then visualised using transillumination by UV light with the UVP 5000 gel documentation system. Analysis by densitometry was carried out as described previously.

2.5.10 Nucleic Acid Fluorescence Assays

2.5.10.1 Microplate-based fluorescence assay for assessment of pure DNA

In preliminary experiments for the assessment of pure DNA, two 50 μL aliquots of each sample were placed onto a microtitre plate. An equal volume of PicoGreen reagent (Molecular Probes, Eugene, OR, USA) diluted 1: 200 in TE buffer was added to each aliquot and incubated for 5 min. The fluorescence of samples was recorded using a Packard Fluorocount Microplate Fluorometer (Packard Instrument Company, Meriden, CT, USA) at an excitation wavelength of 480 nm and emission of 520 nm giving a fluorescence value at pH 8. This was followed by addition of 100 μL of NaOH in the concentration range between 1×10^{-4} and 0.15 M to one aliquot of each sample to attain a final pH ranging from 9 to 13. The other aliquot was left as control to standardise values with respect to pH 8. The identical results obtained between aliquots at pH 8 prompted us to use a single aliquot per sample in experiments. The NaOH solutions were freshly prepared before each experiment from a 10 M NaOH stock. To obtain pH 12.4 in the microwell a 0.1 M NaOH working solution was prepared and further diluted as required to obtain $\text{pH } 12.6 \pm 0.02$, mixing equal volumes of this solution with TE pH 8 gave routinely a final pH of 12.4 ± 0.02 . Under such conditions, a 5 and 10% volumetric increase or decrease of the NaOH working solution shifts the resulting experimental solution by 0.02 and 0.04 units respectively. All measurements were corrected for background fluorescence from a blank containing equal volume of TE buffer, NaOH solution and PicoGreen reagent.

Relative plasmid integrity was determined on Qiagen purified samples using a modification of PicoGreen (Molecular Probes, Leiden, The Netherlands) standard assay described previously (Rock et al., 2003. A copy of this paper, published as part of this project, can be found in Appendix 1).

2.5.10.2 Relative Fluorescence (RF) Assay

The quality of pure DNA was tested using a fluorescence assay based on the rate of unwinding of DNA after the addition NaOH (Rydberg, 1975). In experiments triplicate 50 μ L aliquots of each sample were placed onto a microtitre plate. An equal volume of PicoGreen reagent diluted 1: 200 in TE buffer was added to each aliquot and incubated for 5 min. The fluorescence of samples was recorded using a Packard Fluorocount Microplate Fluorometer at an excitation wavelength of 480 nm and emission of 520 nm giving a fluorescence value at pH 8. This was followed by addition of 100 μ L of NaOH to take the samples to pH 12.4, after 5 minutes the fluorescence was read again and the ratio of fluorescence at pH 12.4 and fluorescence at pH 8 was calculated and is known as relative fluorescence (RF). All measurements were corrected for background fluorescence from blanks containing volumes of TE buffer, NaOH solution and PicoGreen reagent.

2.5.10.3 RF Assay Automation

A four tip Packard Multiprobe II robotic workstation (Packard Instrument Company, Meriden, CT, USA), WinPREP[®] software and integrated plate reader were used. DNA (60 μ L) and PicoGreen (60 μ L) were added to each well in single dispensation per aspiration mode, the 96-well plate was shaken for 5 s in the Fluorocount, incubated for 5 min and read. The plate was transferred to the working station where NaOH (120 μ L) was added via multiple dispensation per aspiration mode, shaken for 5 s, incubated for 5 min and read.

The programme development for the automatic RF assay is detailed within Chapter 7.

2.5.10.4 Fluorescent determination of DNA in impure samples

Samples of supernatant from centrifuged cell suspensions were tested for nucleic acid content using PicoGreen reagent (Molecular Probes, Leiden, The Netherlands) (Singer et al., 1997; Noites et al., 1999). Supernatant samples were diluted 1 in 1000 and 1 in 5000 for greater sensitivity and to decrease signal depression due to interference with proteins and other substances (PicoGreen® dsDNA Quantitation Kit Product Information, Molecular Probes, 2003). 50 µL of diluted samples were placed on a microplate and 50 µL PicoGreen (1 in 400 working concentration) was added and mixed. After 5 minutes the fluorescence of samples was recorded using a Packard Fluorocount Microplate Fluorometer (Packard Instrument Company, Meriden, CT, USA) at an excitation wavelength of 480 nm and emission of 520 nm. The samples were corrected for background fluorescence values using blanks of TE buffer and PicoGreen.

Isopropanol precipitated samples and clarified lysate samples were analysed using an identical method but with 1/200, 1/400 and 1/800 dilutions due to success at these dilutions reported by Levy et al., 2000. Crude cell lysates were analysed by dilution of the cells and lysis mixture and addition of PicoGreen followed by fluorescence analysis as described before. Investigations of RF with impure samples were identical in method to the finished RF assay as described in Chapter 6.

2.5.11 Protein Assay

Samples of supernatant from centrifuged cell resuspension were tested for protein content using Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH, Munchen, Germany). This assay is based on the Bradford assay, using the shift in absorbance of Coomassie Brilliant Blue G-250 dye from 465 nm to 595 nm (Kirazov et al., 1993; Lucarini et al., 1999), although it is not as sensitive as the Lowry assay, it is more suitable for cruder solutions as it more robust to interference (Noites et al., 1999; Singer et al., 1997). Supernatant samples were diluted 1 in 10, 0.8 mL of diluted samples was placed in clean dry cuvettes and 0.2 mL reagent was added and mixed. After 5 minutes the OD at 595nm (OD₅₉₅) vs. reagent blank were read using the BioMate 3 spectrophotometer (Thermo Spectronic, Madison, WI, USA). To obtain protein concentration values, the resulting OD₅₉₅ readings were compared to those obtained from a standard curve of Protein A purified from *E. coli* (Sigma-Aldrich, Poole, UK) chosen because its molecular weight of 45 kDa is close to the average weight of *E. coli* proteins of 40 kDa.

2.5.12 Quantitative Polymerase Chain Reaction (qPCR)

Chromosomal DNA contamination was measured *via* qPCR of an *E. coli* gene. Oligonucleotide primers (Qiagen Ltd., West Sussex, UK) 23S-sense (5' GAA AGG CGC GCG ATA CAG 3') and 23S-antisense (5' GTC CCG CCC TAC TCA TCG A 3') were used to amplify a 70-bp fragment of the 23S ribosomal RNA gene present in the *E. coli* genome as reported in Vilalta et al. The ABI Prism 7700 Sequence Detection System[®] (Applied Biosystems, CA, USA) was used for running the real-time quantitative PCR. 25 µL amplification reactions were applied and each reaction contained 0.5X SYBR[®] Green I Dye (Molecular Probes, OR, USA), 0.025

U/μl AmpliTaq Gold® DNA Polymerase (Roche Molecular Systems, USA), 3 mM magnesium chloride, 200 μM of each dNTP and 0.3 μM of each oligonucleotide primer. 10 ng of plasmid was used per analysis reaction. *E. coli* DH5α genomic DNA was denatured at 95 °C for 15 minutes and cooled with ice prior to use in amplification reactions to generate standard curves from 1 pg to 5 ng.

2.5.13 Rheological Analysis

Samples of cell pastes resuspended with gentle shaking in TE buffer at OD₆₀₀= 50 were analysed using a concentric cylinder viscometer (developed at UCL). Cell pastes were harvested by CSA-1 centrifuge at 50 L h⁻¹ and 250 L h⁻¹; CARR P6 powerfuge at 30 L h⁻¹ and 60 L h⁻¹; and Beckman J2-MI laboratory scale batch centrifuge, using the JA-10 rotor operated at 6000 rpm.

The viscometer was calibrated using polyethelene glycol (MW 5000) before operation. The density of cell resuspension was attained through weighing of 500 mL volumes. Approximately 100 mL of each suspension was poured into the cylindrical vessel and the inner cylinder lowered into the suspension gently. The viscometer was operated and readings of torque and revolutions per minute were taken for six speed settings. The data was then tabulated on spreadsheet and using the width of gap between inner and outer cylinders and length of inner cylinder, shear stress and shear rate were calculated. The viscosity was then calculated from these values and the shear stress curve plotted.

2.6 Referencing of the methods utilised in the following chapters

In each following experimental chapter the methods used will be referenced to this methods chapter. This is with the exception of the method development of the

lysate analysis in Chapter 6 and the algorithms and development of the computer programme for the use of the liquid handling system which is thoroughly described within chapter 7. This is because there is no overlap between these specific methods and those used in other chapters and the detailed pathway of method development is intrinsic to the description of the results.

3 CHARACTERISATION OF PLASMID DNA OBTAINED FROM PILOT PLANT SCALE FERMENTATION AND HARVEST

3.1 Introduction

In this chapter, the effect of 70 and 450 L scale fermentation of *E. coli* on premature cell lysis was investigated. Additionally, the effect of fermentation crash cooling from 37 °C to 13 °C was also assessed for the pQR150 plasmid. Publicly available data on the fermentation performance within the context of plasmid processing is limited to small-scale bioreactors (Boynton et al., 1999, Kay et al., 2003).

The main hypothesis tested was whether the percentage yield of supercoiled (SC) DNA varied with fermentation time, agitation and cooling for fermentations operated at pilot plant scale in the batch mode for 75 L and 450 L bioreactors. During the fermentation, SC plasmid yield was considered as well as the plasmid loss per unit OD at 600 nm (OD_{600}), this allowed an understanding of the shift in cellular plasmid content over the course of the fermentation and past peak harvesting time (peak of exponential growth, usually 7 or 8 hours). The effect of oxygen limitation on cell growth was also considered as well as the steps taken to counter this occurrence.

3.2 Results

3.2.1 Fermentation Kinetics

Initial investigations were carried out at the 2 L scale to evaluate the effect of culture media on the growth of *E. coli* pQR150. Three different media compositions were investigated: (i) Luria-Bertani (1 × LB), (ii) Terrific Broth (1 × TB) and (iii) double strength of Terrific Broth (2 × TB). As shown in Table 3.1, OD_{600nm} for 1 × TB and 2 × TB were at least twice that of 1 × LB. It was decided, however, due to cost limitations, that the cells at pilot-plant scale would be grown on 1 × TB media, despite the increased cell density seen with the use of 2 × TB.

Scale	Media	OD _{600nm} at harvest
2 L	1x LB	5.2
2 L	1 x TB	11.3
2 L	2 x TB	13.5
70 L	1 x TB	25
450L	1 x TB	20

Table 3.1: Comparison of cell density (OD) at small and larger scales using Luria-Bertani (LB) and Terrific Broth (TB). Harvest time for all scales was between 10 and 12 hours.

The growth kinetics were also monitored for inoculation, growth and harvest at the 2 L scale. It was observed that for cells grown on 1 × TB media, the exponential phase ended at 6 h and cells entered the stationary phase whereas the exponential phase continued for the cells grown on 2 × TB media, as shown in Figure 3.1. This feature could be of great importance in the context of large-scale manufacturing as there may be a longer window of harvest if the composition of media were altered.

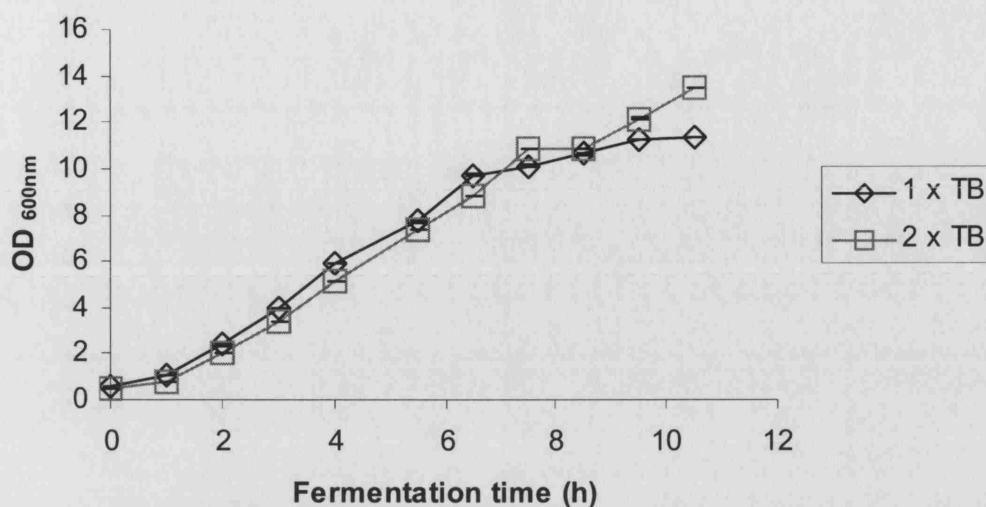
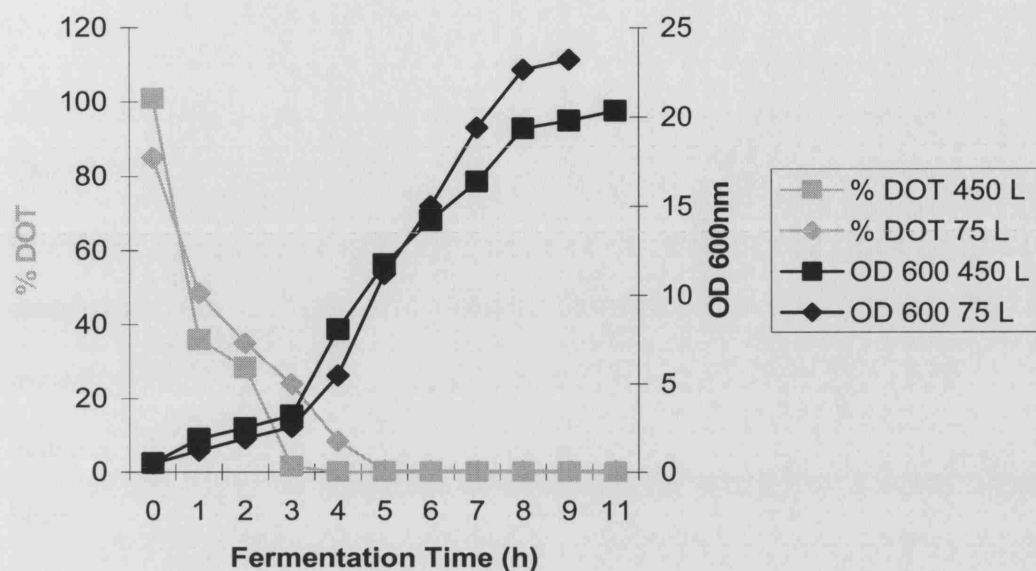


Figure 3.1: Cell growth kinetics for the *E. coli* pQR150 fermentation at 2L shake flask scale. Error bars represent standard deviation of triplicate readings of optical density at 600nm (OD).

Figure 3.2A shows the cell growth kinetics and %DOT data corresponding to 75 L and 450 L scale fermentations of *E. coli* DH5 α containing pQR150. Total plasmid yield and the relative plasmid backbone integrity indicated by the amount of supercoiled DNA (obtained from agarose gel electrophoresis densitometry) were also monitored throughout the 450 L fermentation, results are shown in Figure 3.2B. In contrast, the small scale (2 L) fermentation of the bacterial culture yields optical density no higher than $OD_{600nm} = 14$, as shown in Figure 3.1. This highlights a major difference between cell growths at the different scales; large-scale fermentation gave higher yields due to more efficient oxygen and nutrient transfer.

(A)



(B)

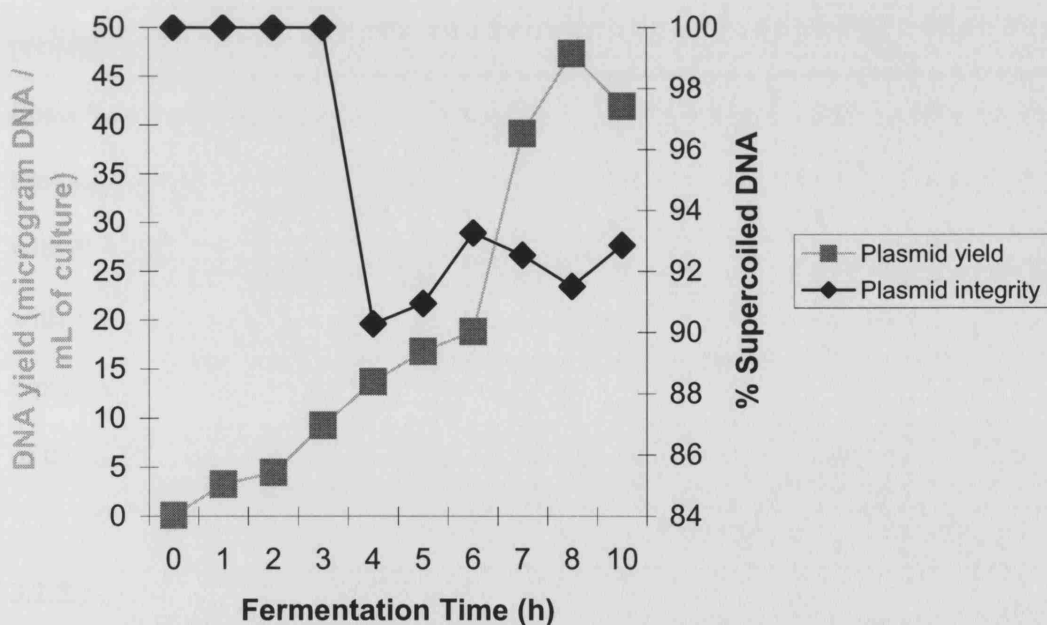


Figure 3.2: (A) Fermentation kinetics: dissolved oxygen tension (% DOT) for 75 L and 450 L cultures, and optical density (OD 600 nm) for 75 and 450 L cultures. (B) Total plasmid yield and relative plasmid integrity throughout the 450 L fermentation. Plasmid yield and relative plasmid integrity (supercoiled DNA) were determined from cell suspension via Qiagen miniprep purification, electrophoresis and densitometry as described in Chapter 2 - Methods and Materials.

3.2.2 Plasmid DNA and protein analysis of cell supernatants

3.2.2.1 Introduction

Once fermentation was completed, spent media was removed, typically *via* continuous centrifugation (see Methods and Materials, section 2.5.3.2) or filtration. The alkaline lysis step was then carried out also described in Methods and Materials. It comprises of cell wall disintegration and then irreversible denaturation of chromosomal DNA; the rheology of this step is complex. The cell suspension is normally Newtonian in nature, however, when cells are broken by the addition of detergent and alkali, the release of the cell constituents cause the cell suspension to become non-Newtonian in nature.

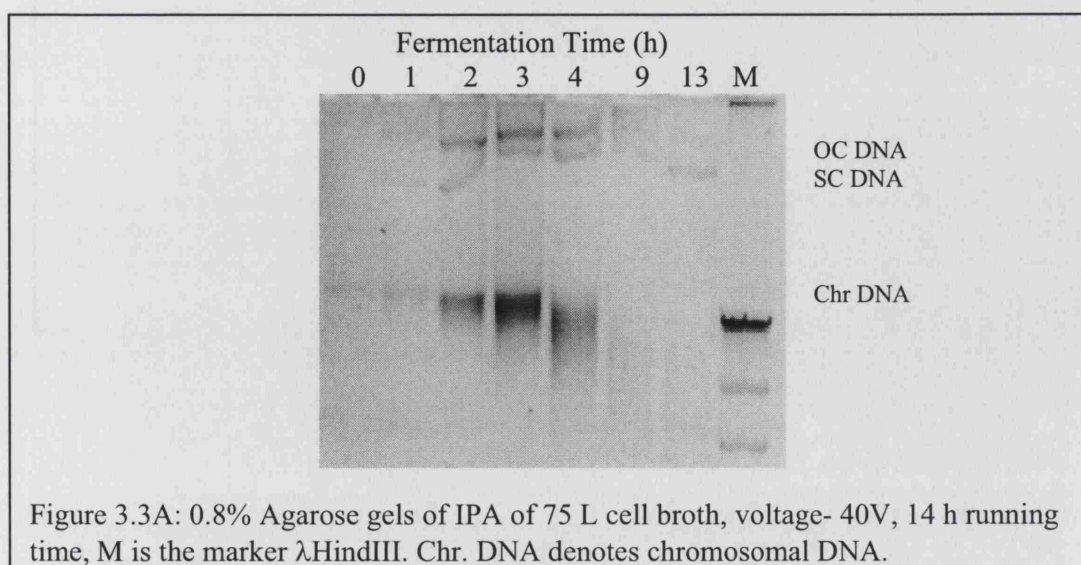
Cells obtained from laboratory-scale fermentations tend to give highly viscous lysates which can be attributed to the relatively long strands of chromosomal DNA present. Upon neutralisation, cell debris and chromosomal DNA form a single flocculent. This flocculent tends to float and separate rapidly from solution. Preliminary tests carried out with cells obtained from 30, 70 and 450 L fermentation with *E. coli* pSV β indicated that viscosity of alkali detergent step varied substantially with fermentation conditions. Upon neutralisation, cell debris and chromosomal DNA typically formed a more particulate flocculent, some of which did not float to the top of the vessel.

3.2.2.2 Analysis of nucleic acid content of cell broth using electrophoresis

The agarose gels of isopropanol precipitated DNA from spent broth confirmed the fragmented nature of the DNA present in the cell broth. Figure 3.3A shows the DNA present in the broth for the 75 L fermentation. There is very little DNA in the 1st hour of fermentation and it is made up entirely of chromosomal fragments. By the 2nd

and 3rd hours of the fermentation, the DNA increases and it contains more, though still very little SC and OC DNA. The mass of extracted DNA increases until the 9th hour, which would usually be near the end of the fermentation, as they are usually harvested after eight hours. After crash cooling which occurred at time = 9h and extended storage at t = 13 h, the release of supercoiled DNA was not significant. When cells enter the stationary phase of fermentation, the cells weaken and become more susceptible to breakage, therefore, it was hypothesised that cooling and storage would have a greater effect than was demonstrated. It is also possible that in addition to greater amounts of breakage there may also have been a greater amount of nuclease activity that may have compromised the electrophoresis analysis in the later stages of fermentation.

The agarose gel of the 450 L fermentation evidenced higher levels of DNA from the 2nd to the 5th hours of fermentation, which again is mainly fragmented in nature (data not shown). Figures 3.3B and C show the approximate mass of DNA calculated from agarose gel electrophoresis and the % supercoiled nature of that extracted DNA. The mass is divided by the OD₆₀₀ reading to give an indication of the



DNA in the supernatant per cell. The highest mass of DNA seen in the gels was 150 ng which gives an extracted mass of 0.00075 g per mL of broth.

It should be noted that this method of densitometric estimation using electrophoresed agarose gels can only be used if special care has been taken to load quantities of DNA that are within the linear range of the densitometric scanning equipment used. The image must also be in focus across the width of the gel. DNA mass values were derived by comparing densitometry in each lane with at least a three point calibration curve with known quantities of a standard sample, which has been Qiagen purified and quantified by absorbance at 260 nm. Preliminary studies indicated that this range was between 30-200 ng DNA per lane. However, even when working within this range a 20-30 % error is associated with this technique.

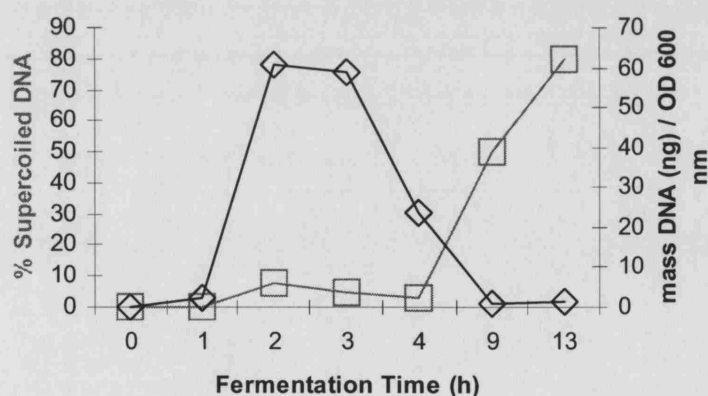


Figure 3.3B: DNA mass per unit optical density (diamonds) and % supercoiled (squares) determined from agarose gel electrophoresis of DNA extracted from 75 L fermentation broth.

3.2.3.3 Nucleic acid content of supernatant of TE cell wash

3.2.3.3.1 Introduction

In order to develop a simple method that could detect evidence of cell lysis a number of experiments were performed, a method that could be used directly on spent broth would be very useful to give real-time information about the status of the culture to the process engineer. The applicability of PicoGreen to these process streams was investigated. The nucleic acid content for the direct cell broth was analysed previously and yielded encouraging results although it was determined that high levels of contaminants present in the broth may cause interference with the PicoGreen signal. Cell pastes were washed by re-suspending the cell paste in TE buffer and centrifuging the cells at the laboratory-scale. The supernatant from this wash was then analysed for nucleic acid and protein content using PicoGreen as well as traditional methods. This washing method removes some of the contaminants known to affect both the standard PicoGreen and Bradford protein assays.

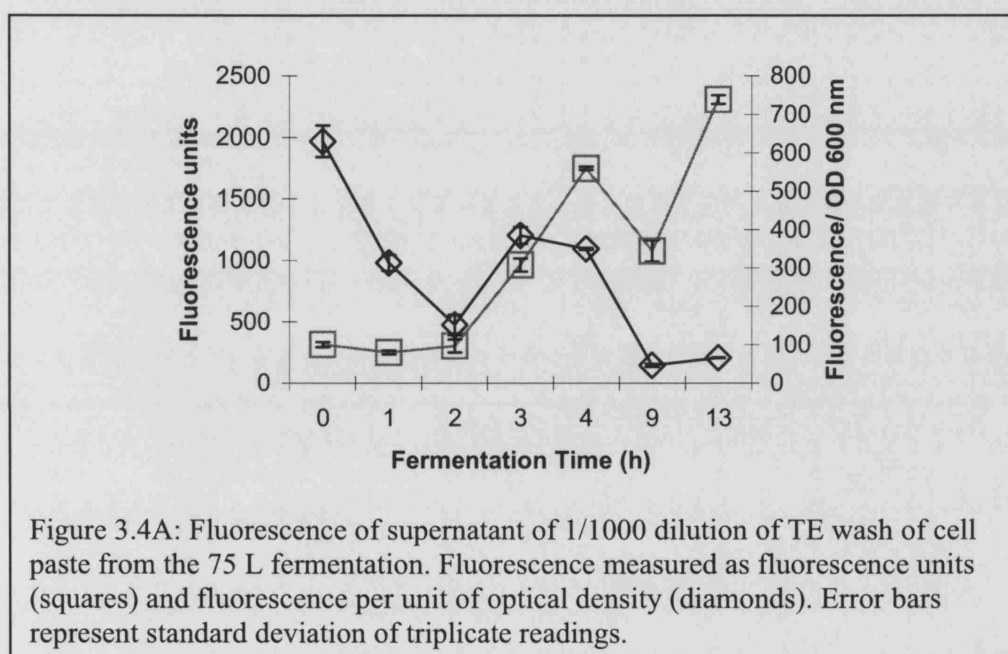
The contaminant that may cause the highest degree of interference is protein, we know that the typical *E. coli* cell is 55% protein, seen in Table 1.3 in Chapter 1. The weight of one cell is around 7.5×10^{-13} g with about 70% of this mass being water. The typical *E. coli* overnight fermentation yields about 1.5 g per 500 mL and in rich media such as was used for this project, yield may be increased to 3 – 4 g per 500 mL, seen in data gained over the course of the project. The cell mass from 500 mL fermentations is centrifuged and resuspended in 100 mL TE buffer for plasmid extraction. From the alkaline lysis method the 100 mL resuspension is made up to 300 mL with lysis and neutralisation solutions; see Methods and Materials. Taking 4 g as the cell weight in this solution, at 70% water, the cell debris and contents weight

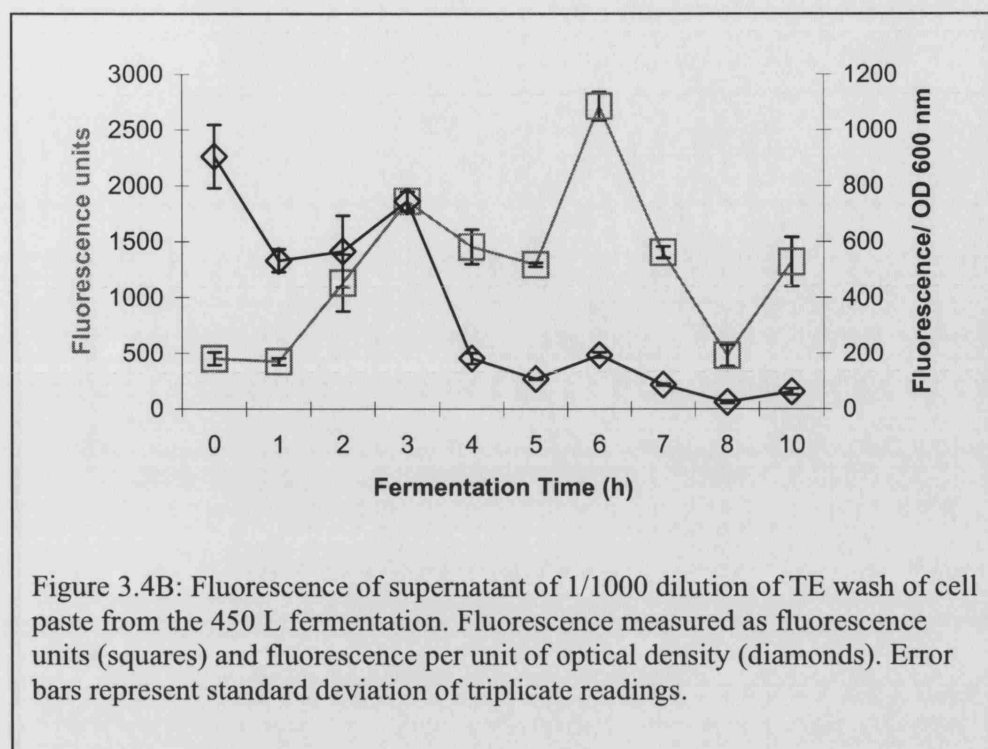
(without water) is 1.2 g. Within this neutralised crude lysate solution, protein represents 0.66 g in 300 mL. Thus, protein is 0.22% of the total weight of the crude lysate. This level would be even lower in a crude cell broth where cells are mostly unbroken or in a TE wash of bacterial cells. Crude cell suspensions and lysates are diluted to 1/400 – 1/1000 before PicoGreen analysis. Given that the level of protein is 0.22% in the typical lysate used within this project it may be seen that the dilutions used for testing are well below the levels seen to cause interference from the manufacturer's data shown in Table 1.5 and in Singer et al., 1997).

3.2.3.3.2 Direct Analysis of DNA in supernatant wash using PicoGreen

In previous studies, washing cell paste with TE buffer was found to be a useful step in DNA extraction that removed chromosomal and plasmid DNA fragments from the product stream. This DNA would have been released from cells primarily damaged during primary recovery. The samples collected from the 75 and 450 L fermentation were harvested using a bench top microcentrifuge so that the damage inflicted on the cells would be less than that seen with the disk stack centrifuge in Chapter 4. However there would still be some cells broken during harvest, as well as residual DNA from cells broken during the fermentation.

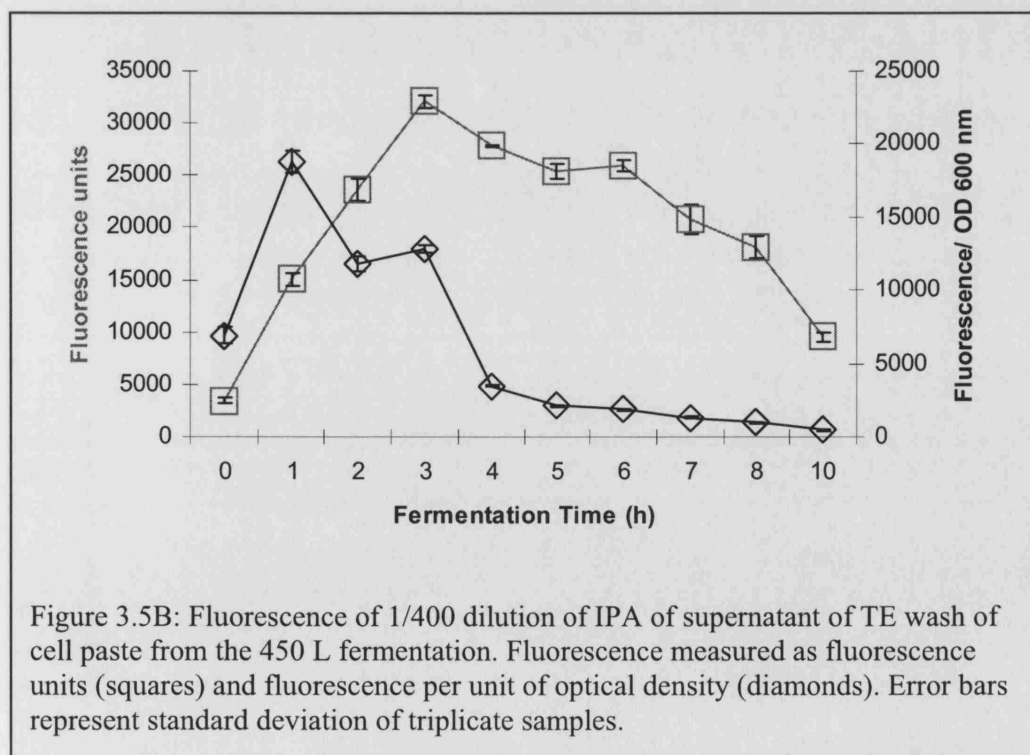
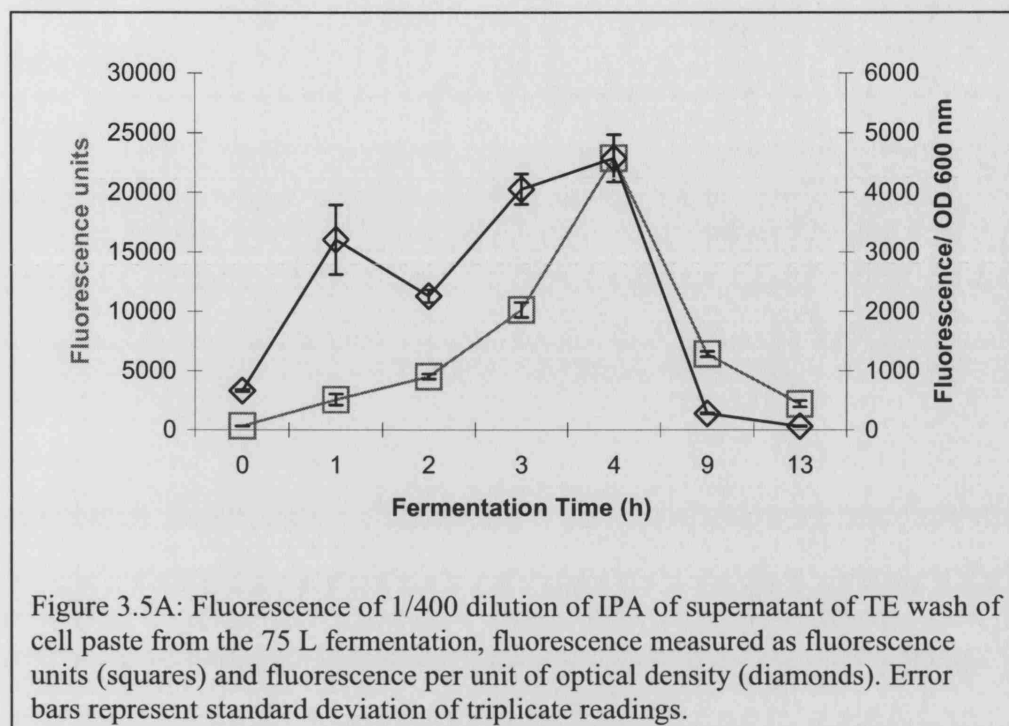
Figure 3.4A shows the fluorescence of the TE wash over the course of the 75 L fermentation. As the cell density increases the DNA released in the supernatant decreases, except for a spike at 3 hours. There is also a small increase between 9 and 13 hours after the commencement of crash cooling. The spike at 3 hours and slight increase after 8 hours are also seen with the 450 L fermentation, Figure 3.4B. This could suggest, that there was either an increase in DNA mass released or DNA degradation that coincided with that time period.



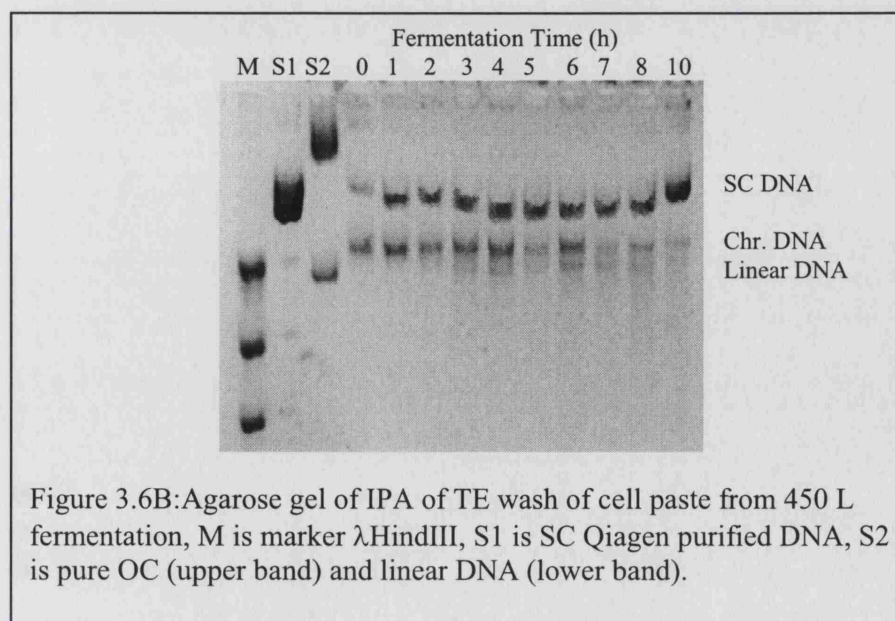
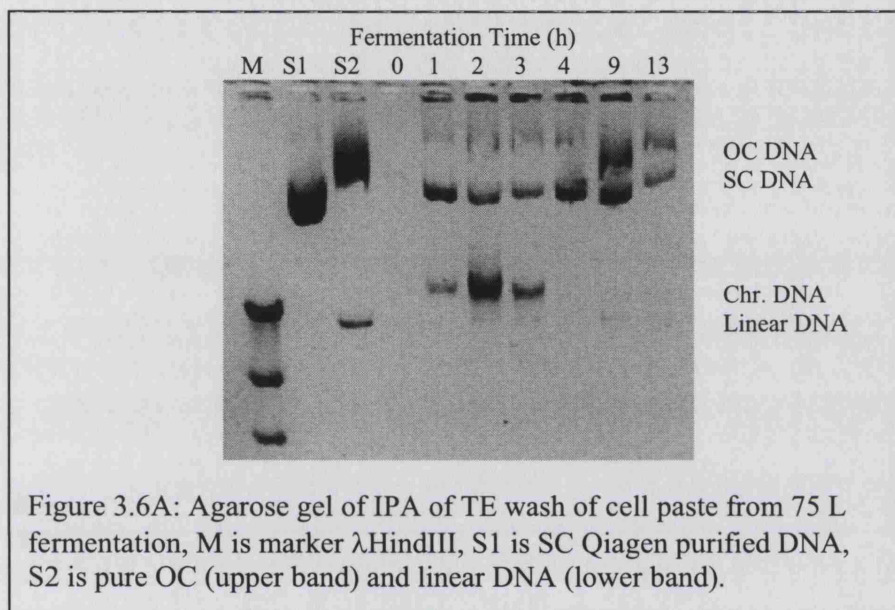


3.2.3.3.3 Comparison of isopropanol precipitated DNA extracted from TE wash and from cell broth

DNA extracted from the supernatant followed a similar trend to direct analysis of the supernatant wash with PicoGreen but with peaks at time =1 and 3h, Figure 3.5A. However, the value at the time of inoculation ($t = 0$) was much lower suggesting that either the PicoGreen test on direct supernatant wash may have given a falsely high reading, due to broth conditions at that time, or conversely, that any DNA present at this time was too degraded or fragmented to precipitate well. Degraded DNA also gives a very high reading. Similar general trends were seen with the 450 L fermentation, Figure 3.5B.

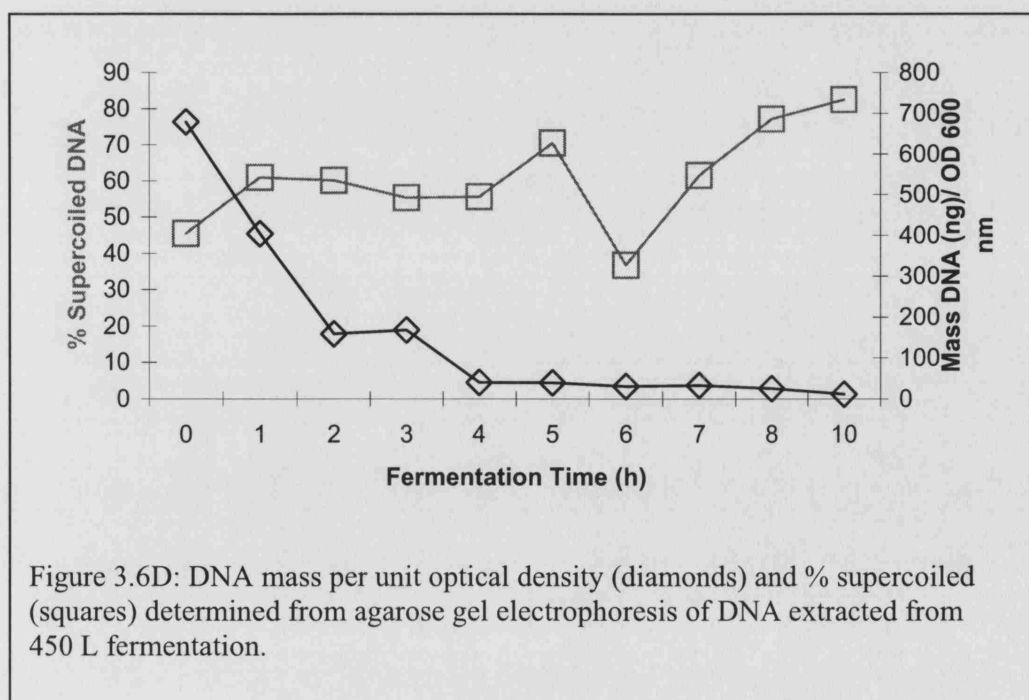
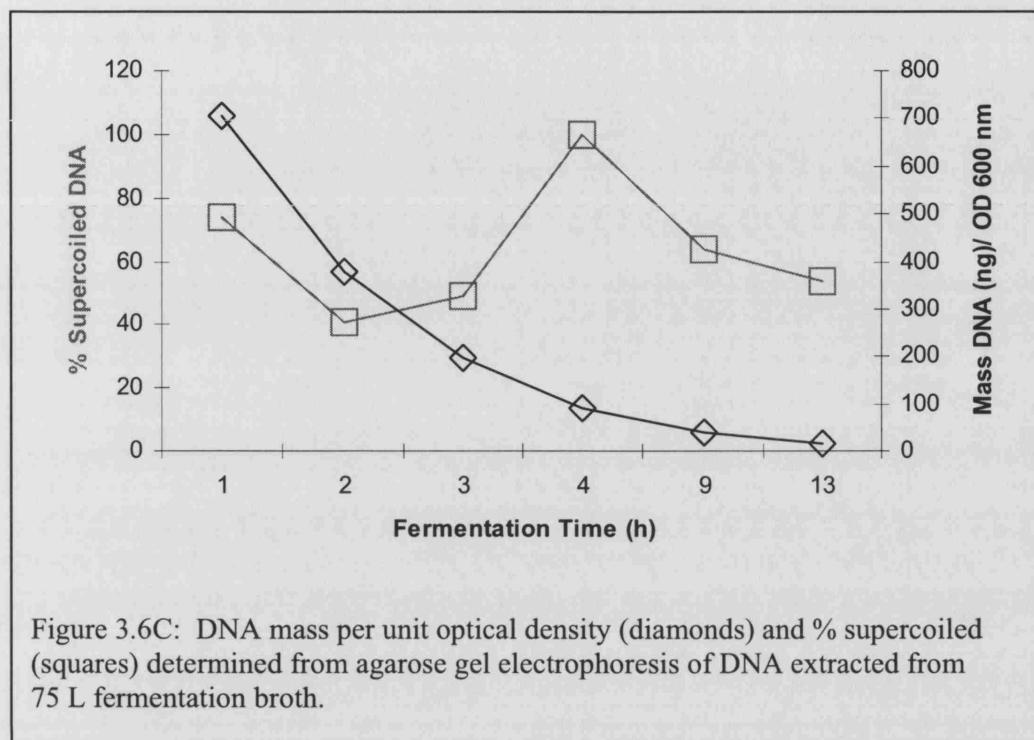


From Figures 3.6A and B it may be seen that there are some significant differences between the 75 and 450 L fermentations. The early hours of the 75 L fermentation show significant chromosomal contamination which is not apparent beyond time = 3 h. There are also significant amounts of open circular DNA present which rises throughout the fermentation until a drop at time = 13h.



By contrast there were no open circular forms seen in the gel for the 450 L fermentation although there is significant evidence of small fragments of DNA which show as smears just under the chromosomal band. This DNA may be chromosomal or plasmid in nature, and this explains why higher fluorescence was seen for the 450 L, Figure 3.5. However, the presence of significant fragments means that the mass of DNA estimated from gel electrophoresis using densitometry is probably underestimated, Figures 3.6 C and D.

The masses of DNA estimated from each lane divided by the OD_{600} at each time point are shown in Figures 3.6 C and D. DNA mass decreased over the course of the fermentation. Percentage supercoiled for the 75 L fermentation varied more than for the 450 L, with average supercoiled content of 63.4 ± 21.2 % compared to 60.3 ± 13.7 %. The lower variance of the 450 L means that the fluorescence trend is far more reliable and unlike the correlation seen with the DNA extracted from cell broth, the correlation here is much better, with coefficient $R^2 = 0.91$ (data not shown).



Further experiments may be required to evaluate the significance of the level of cell lysis observed in the whole process. In the manufacturing process, specifications on the maximum cell lysis accepted can be used to obtain a more in-depth insight into the fermentation process.

Figure 3.7A shows the fluorescence with fermentation time for 1/400 dilution of the isopropanol precipitated DNA (IPA). The trend roughly followed that seen with the direct testing with the highest levels of fluorescence seen at the time = 1-2 h. For the IPA of 75 L fermentation samples, Figure 3.7B, the highest figures of fluorescence occurred at the 3rd hour of fermentation, and decreased markedly after the 5th hour. The fluorescence levels were also comparable with those seen during direct supernatant testing. For the 75 L, values were slightly higher than for the 450 L indicating that there may have been some loss of DNA during the precipitation of DNA from the broth of the 450 L fermentation which may have been affected by the probable fragmented nature of the DNA in the broth. The fluorescence, at appropriate dilution of DNA extracted from Terrific broth, was 3000 Fluorescence units (FSU). Despite losses occurring during precipitation the trend of fluorescence decrease is similar.

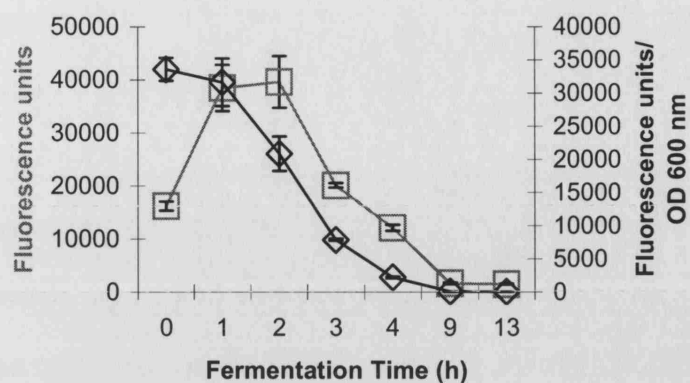


Figure 3.7A: Fluorescence response of Isopropanol precipitated DNA (IPA) of cell broth of 75 L fermentation at 1/400 dilution. Fluorescence measured as fluorescence units (squares) and fluorescence per unit of optical density (diamonds). Error bars represent standard deviation of triplicate readings.

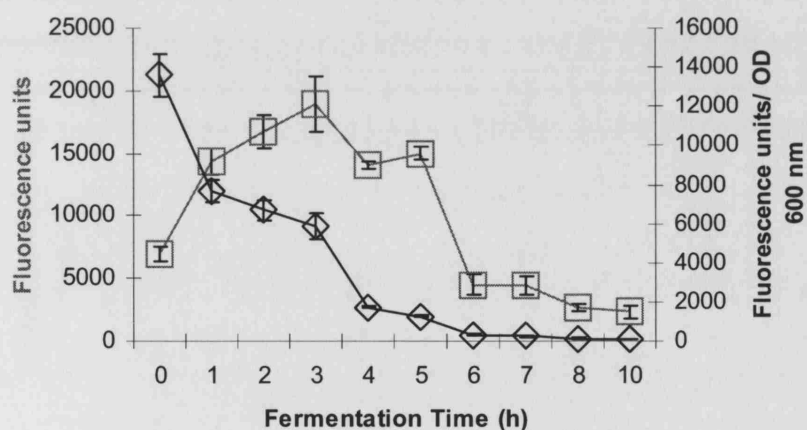
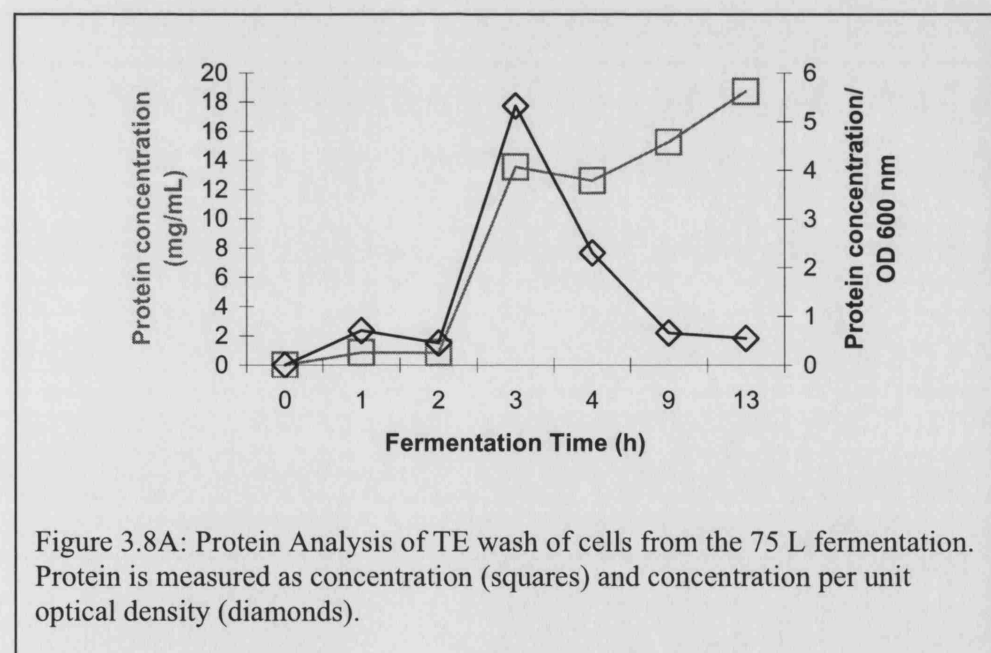
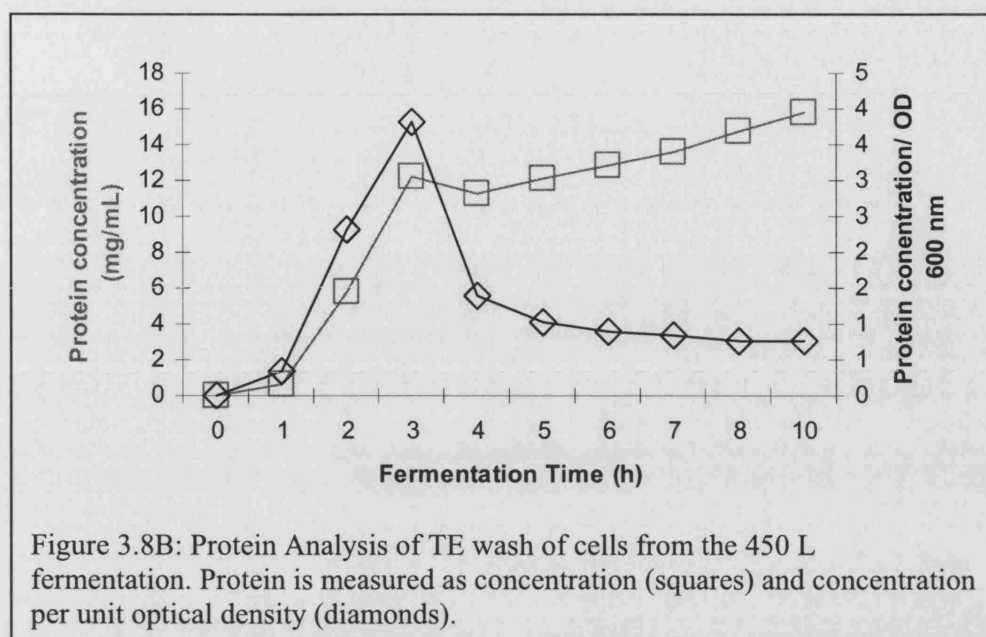


Figure 3.7B: Fluorescence responses of IPA of cell broth of 450 L fermentation at 1/400 dilution. Fluorescence measured as fluorescence units (squares) and fluorescence per unit of optical density (diamonds). Error bars represent standard deviation of triplicate readings.

3.2.4 Protein Content of Supernatant using the Bradford Assay

Analysis of the supernatant by protein confirms cell breakage in an independent manner. Protein estimation of the cell wash from the 75 L fermentation (Figure 3.8A) shows peaks at 1 and 3 hours whereas for the 450 L fermentation, the main peak was seen at 3 hours, Figure 3.8B. The question is what has happened in the 3rd hour of fermentation to cause such a disproportionately high level of DNA and protein release. To compare to Figure 3.2, showing fermentation kinetic data, by the 3rd hour of both fermentations, it had become apparent that cell growth was outstripping the capabilities of the fermenter. Percentage dissolved oxygen tension was 0 for the 450 L and 22 % soon to decrease to nil. This decrease in oxygen was countered by an increase in airflow rate and agitation, in order to increase cell oxygen uptake but in the end this may have been damaging.





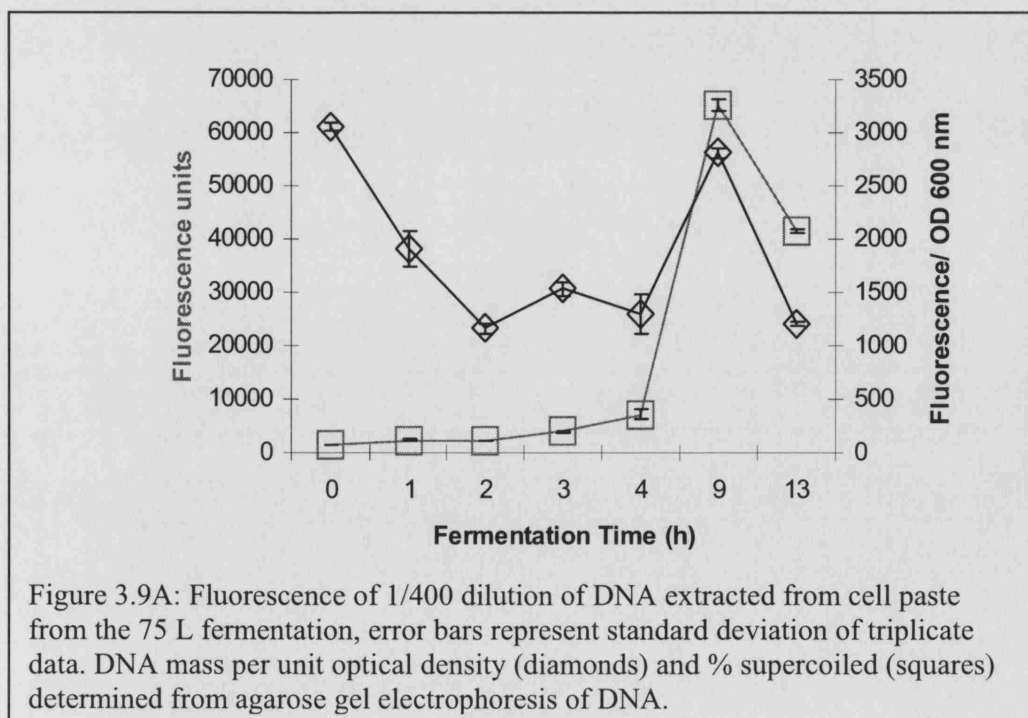
3.2.5 Nucleic Acid and Protein Content of Cell Pellet

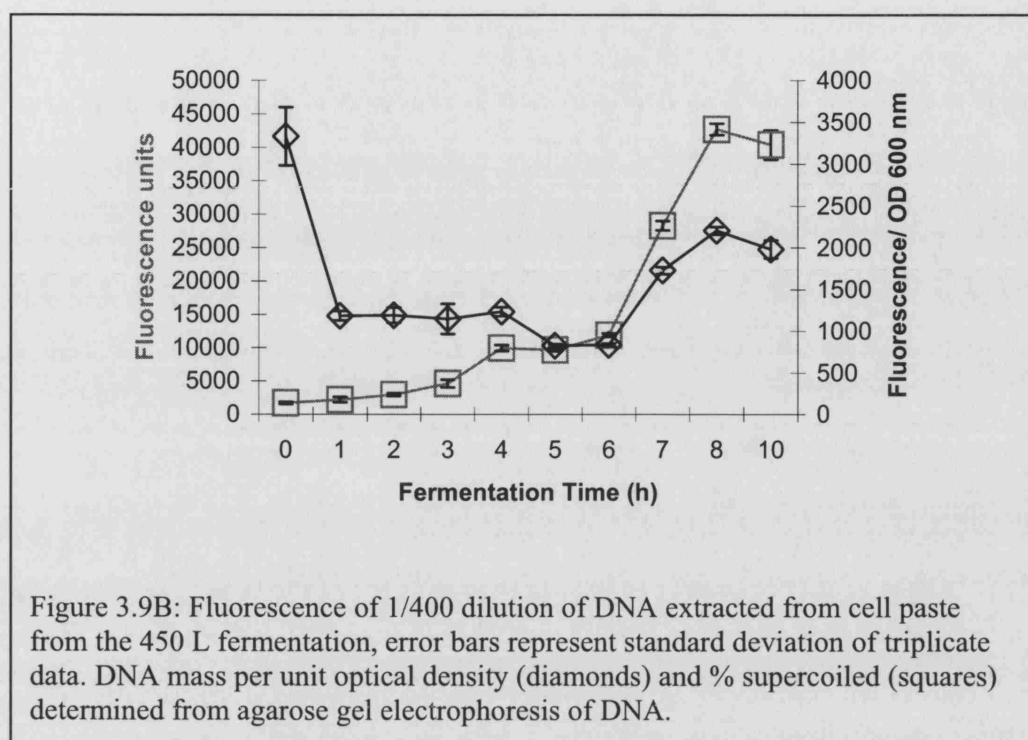
3.2.5.1 Nucleic Acid Content of Isopropanol Precipitated DNA

The product of the process, the plasmid DNA, lies within the bacterial cell, thus, analysis of this part of the process stream is most important for assessing which conditions truly affect the yield and purity of the product and to what degree. Isopropanol extraction of DNA is an important step for concentrating DNA before loading onto a chromatography column especially when purifying large plasmids (Wade-Martins, 1999). Firstly, PicoGreen was used to assess the nucleic acid content of a 1/400 dilution of the rough isopropanol extract of cells, Figures 3.9A and B. The most notable aspects of the fluorescence reading are the high values seen at the start of the fermentation and the drop seen after 9 h for the 75 L and 10 h for the 450 L fermentations. The pQR150 plasmid is a high copy pUC based plasmid that maintains a copy number of around 50-100 per *E. coli* cell. Although the number of copies per cell should not vary over the course of the fermentation, when the cells are rapidly dividing, the number of plasmid copies that they hold will be less than during the

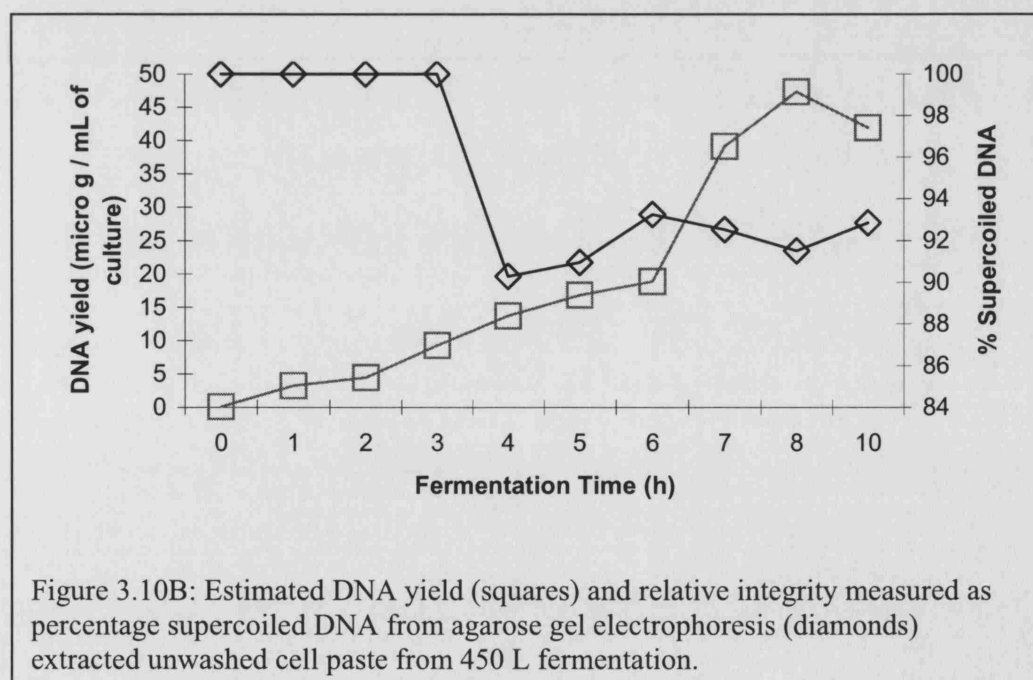
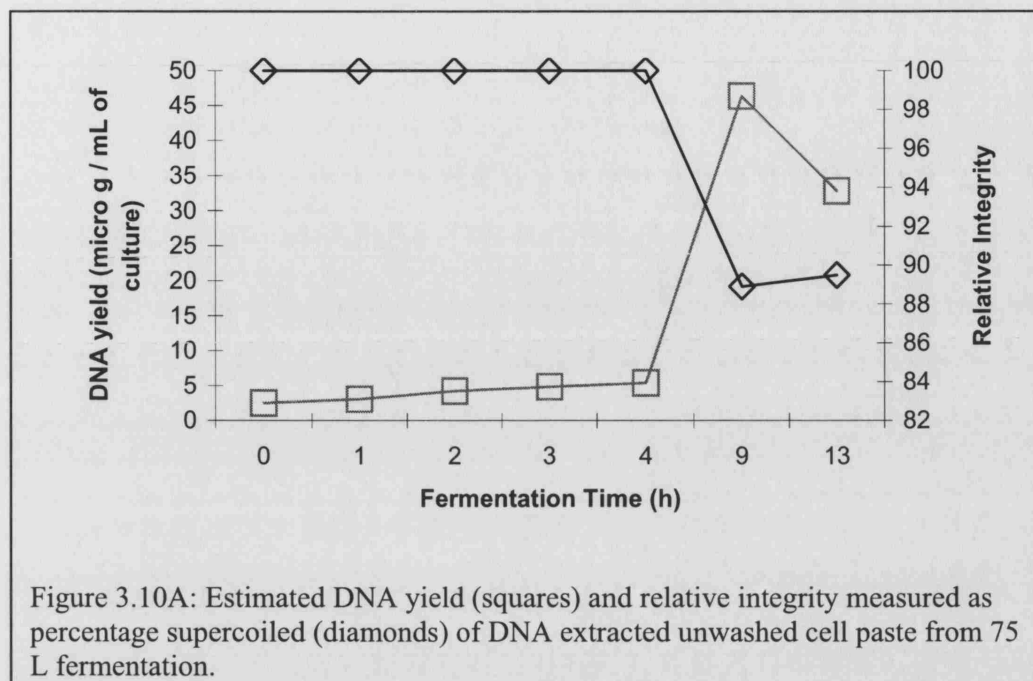
stationary phase. However, during the stationary phase the plasmid has to contend with the cell degradative processes which lead to death, thus the need to harvest at the peak of exponential growth.

Figures 3.9 A and B show the data obtained from the measurement of fluorescence of IPA DNA and PicoGreen. The high fluorescence at the start may be indicative of the mature fermentation that was used to inoculate the large-scale culture. This is due to the heightened PicoGreen fluorescence signal seen with degraded plasmids (Levy et al., 2000). From looking at the growth curve for the fermentations, Figure 3.2, it can be seen that stationary phase occurs at time = 12 and 8 h for the 75 and 450 L fermentations, respectively. The fluorescence readings seem to indicate that there may have been degradative processes occurring after $t = 9$ h, although this was not suggested by analysis carried out previously using agarose gel electrophoresis.



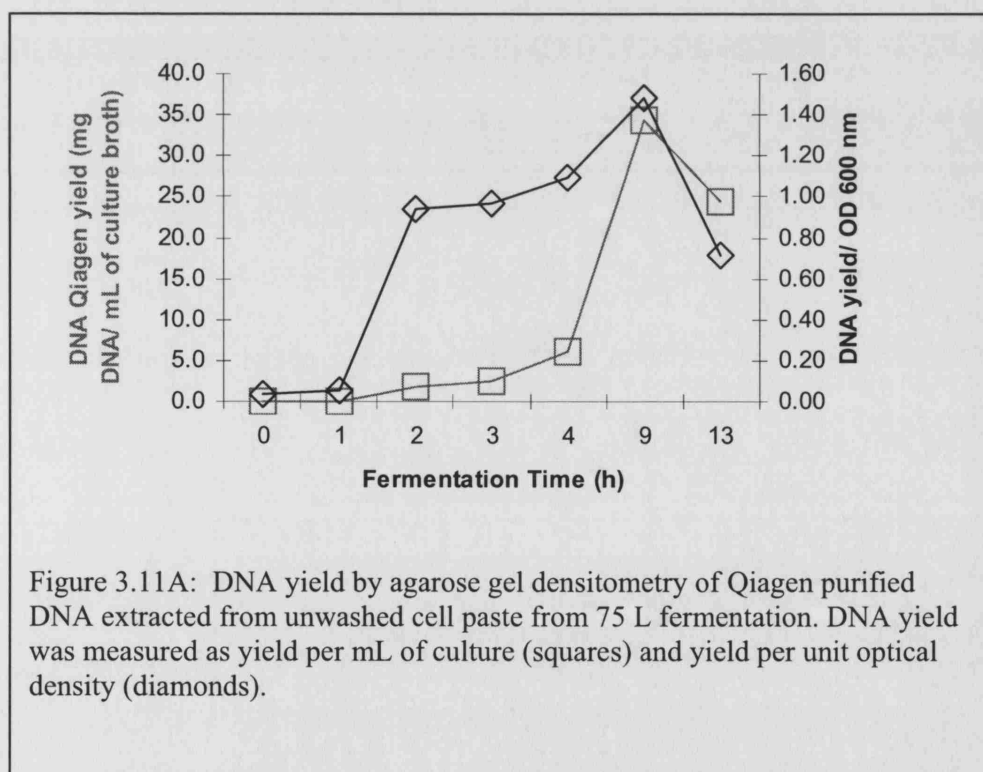


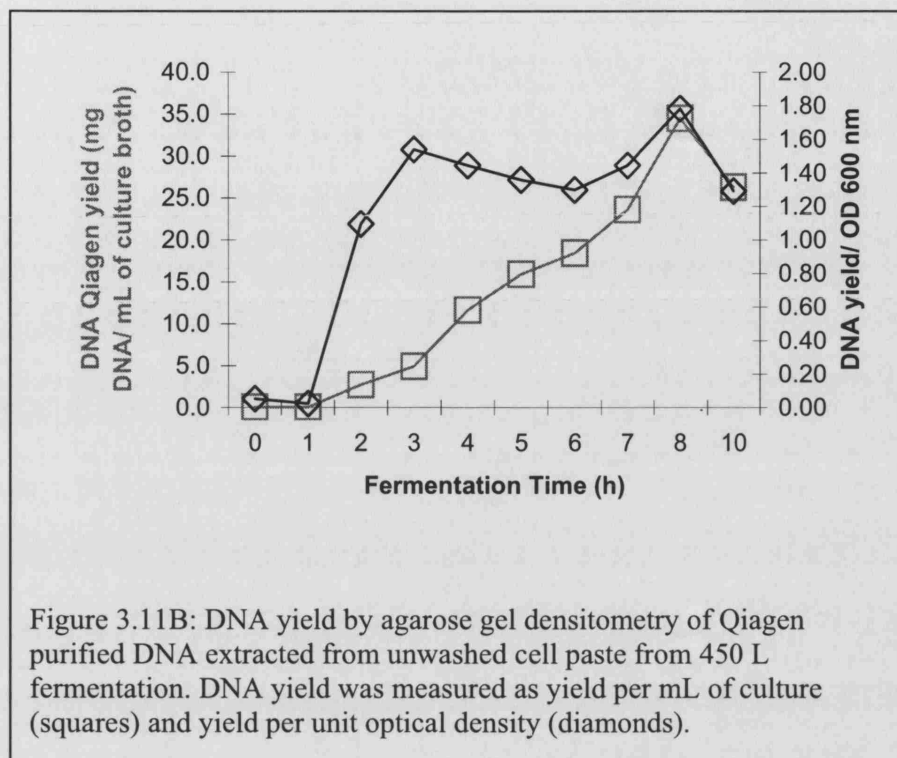
Figures 3.10A and B show the yield of DNA (as mass of DNA extracted per mL of cell culture) obtained by gel densitometry, again yield is highest at 8 and 9 hours for the 450 and 75 L fermentations, respectively and drops by 11 and 29% after these time points. DNA yields per OD₆₀₀ unit are at their highest at the start, are lowest in the fastest growing segment of the fermentation rise until time = 8 and 9 h for 450 and 75L, respectively and drop after these time points. The condition of the extracted DNA also varies over the course of the fermentation, the DNA extracted from the 75 L fermentation has a 100 % supercoiled character, as estimated using agarose gel electrophoresis until the time = 9-13 h when it falls to 90%. In addition, supercoiled falls at time = 4 h from 100 to 90% for the 450 L fermentation but rises again to 93% by the end of the fermentation. Statistically these decreases were not significant but the observations warrant further investigation.



3.2.5.2 Plasmid yield and integrity for Qiagen Purified DNA

The final part of the analysis of the cell suspension was the investigation of extracted plasmid DNA. Using Qiagen mini chromatography columns, plasmid content of cells, the eventual yield and quality of the plasmid change over the course of the fermentation were investigated. It may be seen in Figure 3.11 A and B that DNA yield decreased after 9 and 8 h for the 75 and 450 L fermentations, respectively. Again, this indicated that there may have been some degradative processes occurring at this time point. The purity of all DNA extracted by Qiagen was high absorbance ratio $A_{260}/A_{280} = 1.8-1.9$.





3.3 Conclusions

The fermentation of *E. coli* pQR150 in 1 × TB was successfully scaled from laboratory to 70 and 450 L and high cell densities were achieved. The plasmid yield achieved was in agreement with values reported by others (Levy et al., 2000).

The assay used to assess premature cell lysis *via* direct measurement of plasmid released using the PicoGreen dye showed a release of DNA from cells that coincided with an increase in agitation and airflow rate that was carried out in response to the decrease in dissolved oxygen tension. Further studies should be carried out to assess the significance of this observation and to quantify accurately the detrimental effect of operating conditions on yield. Crash cooling was found not to significantly impact the plasmid yield and quality through analysis by agarose gel electrophoresis. However, decreases in DNA yield at this time were observed, indicated by analysis of cell pellet, and warrants further investigation.

Direct measurement of DNA in process streams using fluorescent dyes, as was demonstrated by the TE wash in this chapter, can be used to monitor cell status before transferring between vessels and after holding periods which are sometimes inevitable when working at the large-scale. Direct measurement of DNA in streams associated with the spent broth (such as the TE wash) could be used as an enabling tool to address whole bioprocess sequences and eliminate any interactions between engineering conditions that impact on plasmid product yield. This should be further validated and assessed for suitability as a process analytic technique with possible industrial application.

4 CHARACTERISATION OF BACTERIAL CELL PASTES FROM PILOT PLANT SCALE FERMENTATION AND HARVESTING

4.1 Introduction

The growing importance of the precise definition of the properties of plasmid process streams is a counterpart to the development of DNA therapies and the increasing scale of the plasmid production process seen in the development of these new therapies. As methods move from the laboratory to the pilot plant and then on to the industrial scale there is a need to develop a full understanding of the impact of the large-scale processing equipment on the plasmid stream. Ensuring that process streams are of identical composition at all scales is the over-riding consideration for the plasmid pharmaceutical process in order to ensure the high output quality of product at the end of purification. Identifying these characteristics is particularly important in today's drug manufacture industry where some fermentation may be outsourced for economic reasons but with the cell paste purified and the product formulated in-house. In large companies, poor communication between the fermentation and downstream processing staff can also impact on plasmid reproducibility. There is also the natural limitation of process scale, it is practically impossible to process all cell paste resulting from large-scale fermentation immediately and some paste must be held, usually by freezing, this is then thawed as necessary for purification. Scale is again of particular importance in the lysis step where the prime consideration is for the efficient mixing of cells and lysis buffer. A lysed cell suspension has a high viscosity and combined with limited holding time results in a process stage that is in effect scale limiting.

Most large-scale plasmid DNA processing strategies developed from well-established laboratory scale protocols (Schleef et al., 2002; Levy et al., 2000; Prazeres et al., 1998; Murphy et al., 1999). Typically cells are harvested at late log or stationary phase *via* centrifugation, resuspended in an appropriate buffer and chemically lysed (O’Kennedy et al., 2003). The alkaline lysis procedure entails the use of alkali and detergent followed by neutralisation with, for example, concentrated potassium acetate. The alkali-detergent mixture causes irreversible denaturation of chromosomal DNA and proteins and, providing pH is not too high, reversible denaturation of the plasmid DNA. Plasmids are, therefore, soluble in the neutralised solution whereas most chromosomal DNA and cellular debris form an insoluble flocculant.

The lysis step is seen as one of the most critical operations in large-scale plasmid DNA processing as any fragments of chromosomal DNA that remain in solution will have a detrimental effect in downstream operations (Levy et al., 2000; Levy et al., 1999b). Previous studies have investigated the effect of fluid mechanical force during the lysis step using a rheometer and stirred reactors (Ciccolini et al., 2002; Chamsart et al., 2001) and shown that cell lysate is susceptible to shearing forces that negatively impact further downstream processing.

In this chapter, the effect of bacterial cell centrifugation and resuspension conditions on the initial stages of plasmid DNA processing was investigated. *E. coli* DH5 α cells containing a 20 kb plasmid pQR150 were grown in a 450 L batch bioreactor. Cells were recovered by centrifugation using either a disk stack or a tubular bowl centrifuge operated at flow rates between 30 and 250 L h⁻¹. It is hypothesised that under the operating conditions used, cells harvested with the two large-scale centrifuges will produce alkaline lysates containing higher levels of

chromosomal and degraded plasmid DNA contamination than lysates produced using laboratory scale equipment. Likewise, the length, temperature and agitation of the cell resuspension operation will also impact on product yield and the levels of DNA contamination in the lysate. Direct measurement of released DNA using PicoGreen at the cell resuspension stage will be evaluated as a simple process analytical technology suitable for development and manufacture.

A paper concerned with this chapter has been submitted for publication, a copy of which can be found in Appendix 2.

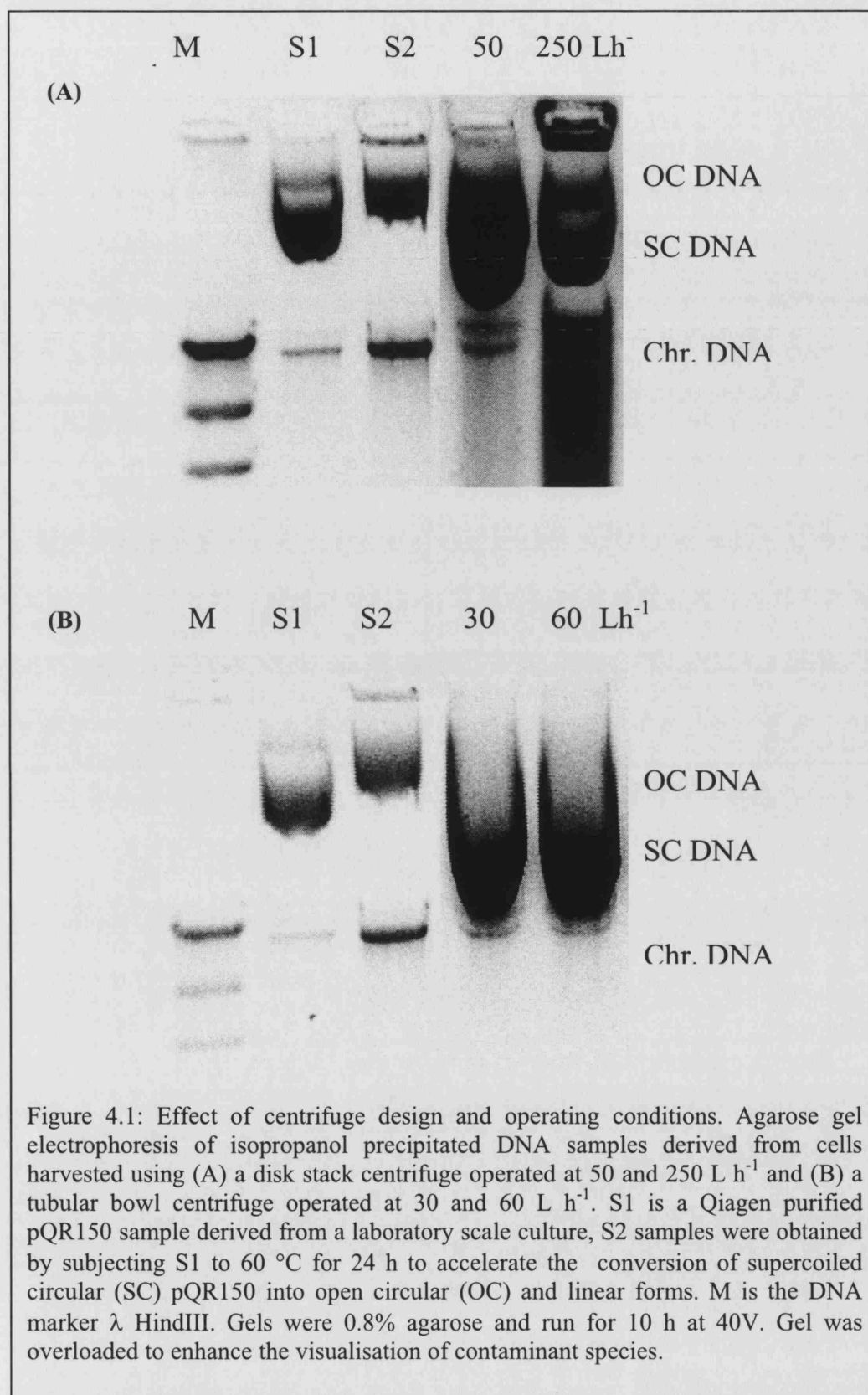
4.2 Results

4.2.1 Analysis of plasmid DNA extracted from fresh and frozen cells

The cell growth kinetics and percentage DOT data corresponding to a 75 L and 450 L scale fermentations of *E. coli* DH5 α containing pQR150 are shown in Figure 3.2A. Total plasmid yield and the relative plasmid integrity (average integrity of plasmid molecules in the sample) were also monitored throughout the 450 L fermentation and results are shown in Figure 3.2B. The entire culture was then harvested using two different pilot plant scale continuous centrifuges: the CARR P6 tubular bowl and the CSA-1 disk stack operated at flow rates of 30 and 60 and 50 and 250 L h⁻¹ respectively (Boychyn et al., 2000; Boychyn et al., 2001). The fresh cell pastes obtained (kept at 4 °C for no more than 4 h) were resuspended in TE to OD₆₀₀ = 50 and subjected to the alkaline lysis procedure. The lysates were clarified by filtration, as described in Methods and Materials. Isopropanol precipitated (IPA) DNA extracted from cells derived from the disk stack or the tubular bowl centrifuge was

analysed *via* electrophoresis. The cells harvested with the disk stack centrifuge produced a lysate that showed a smear of low molecular weight contaminant DNA species, the levels of which increased more than 2 fold with increasing flowrate (Figure 4.1A). By contrast, this contaminant DNA smear was not evident in the material derived from the tubular bowl and no differences in the DNA profile were observed for the two flow rates studied (Figure 4.1B).

Physical examination of the cell sludge upon harvest and resuspended cells ($OD_{600} = 50$) produced from the cell pastes determined that the cell paste harvested using the disk stack centrifuge was of a more sticky and viscous quality than cell paste harvested by the tubular bowl centrifuge which was also more sticky and viscous than cells harvested by the laboratory scale batch centrifuge. This quality was confirmed through analysis using a concentric cylinder viscometer and estimation of the viscosity of resuspended cells. Rheological analysis of the suspensions of cells harvested using the disk stack and tubular bowl centrifuges showed that the suspensions were non-Newtonian in nature as the shear stress did not rise proportionally with shear rate, in fact, shear thinning properties were observed. This indicated that significant breakage of the cells had occurred prior to resuspension. Additionally, within the viscometer, the applied torque was observed to drop if the sample was left under a constant shear rate over a time period of order 1 minute. Consequently, the peak torque was used in the preceding analysis. In contrast, the suspension of cells harvested using the JA-10 laboratory scale centrifuge showed Newtonian properties whereby the shear stress exhibited by the fluid was proportional to the shear rate applied. This is likely to be due to the lower breakage of cells in the laboratory scale centrifuge. The estimated viscosity of the JA-10 cell suspension was $7.8 \text{ N m}^{-2} \text{ s}$.



A series of experiments were performed in order to evaluate whether the differences observed between the disk stack and the tubular bowl derived material could be attributed to either the method of the cell harvest, scale of lysis process or the clarification method. Poor mixing of cells and lysis buffer at larger scales may lead to isolated regions of very high pH which may result in irreversible denaturation and thus lower yields of plasmid product (Ciccolini et al., 2002). Inefficient mixing of cell lysate and neutralisation buffer may lead to regions exposed to high pH for longer than acceptable periods of time also leading to reduced plasmid product yields. Clarification method was investigated through the use of traditional dead-end filtration using Whatman no.1 paper and laboratory scale batch centrifugation using the JA-10 rotor. It is generally accepted that filtration is a more gentle method of flocculant isolation which leads to less breakage of flocculant. Filtration is particularly preferred for extraction of bacterial artificial chromosomes. Lysis flocculant is composed of chromosomal DNA and cell debris and if disturbed during the lysis process leads to higher levels of chromosomal DNA contamination in the plasmid product and issues of co-purification of fragmented *E. coli* chromosomal DNA further downstream.

Frozen cells from the disk stack and tubular bowl centrifuges were thawed, resuspended in buffer to $OD_{600}=50$ and subjected to the alkaline lysis procedure at 0.5 and 300 ml scale. The flocculant (cell debris, proteins and chromosomal DNA) was removed by either batch centrifugation or dead-end filtration using Whatman no.1 paper. DNA was isopropanol precipitated from the different clarified lysates obtained and was loaded onto agarose gels for analysis. Results, shown in Figure 4.3, confirmed trends observed for fresh cell paste material (Figure 4.1). Furthermore, they indicated that under the conditions used, neither the scale of lysis nor the clarification

method had a substantial impact in the profile of contaminant DNA species as analysed by agarose gel electrophoresis. Densitometric scanning of the gels confirmed again that samples derived from disk stack centrifugation operated at 250 L h⁻¹ had significantly increased levels of low molecular weight contamination than the 50 L h⁻¹ samples.

It was observed that all lysates derived from the disk stack centrifuge showed a distinct contaminant band that migrated in between the SC and the linear form of the plasmid (Figure 4.1A and 4.2B). The identity of this band was further confirmed by a control experiment using the parental DH5 α host strain. Results indicated that this band could be attributed to host derived chromosomal DNA (Figure 4.2C). This result is also in agreement with southern blot analysis using a specific probe for *E. coli* chromosomal DNA reported previously by Levy et al., 1999.

Cells harvested with the laboratory scale batch centrifuge yielded 5 μ g DNA per mg dry cell weight, had a plasmid supercoiled content of 54% and after ion exchange purification had a remaining of 4% w/w chromosomal contamination, as assessed by qPCR. By contrast, the material harvested with a disk stack centrifuge operated at 250 L h⁻¹ had 39% w/w chromosomal contamination, as assessed by qPCR. The performance of the tubular bowl operated at 60 L h⁻¹ was in between the one of the disk stack and the batch centrifuge (data supplied by Simyee Kong, University College London). This data is shown in Table 4.1.

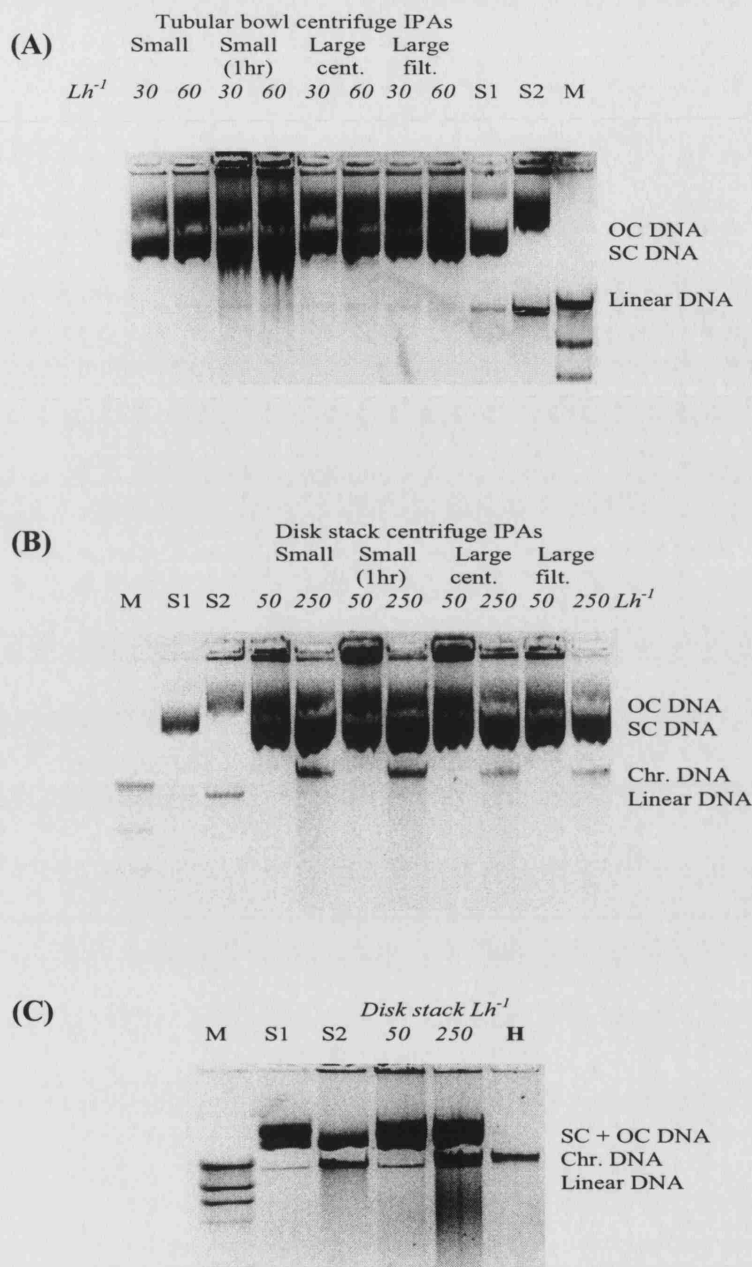


Figure 4.2: Effect of lysis scale and clarification procedure. Agarose gel electrophoresis of isopropanol precipitated DNA (IPA) samples derived from cells harvested using (A) a tubular bowl centrifuge operated at 30 and 60 Lh^{-1} and (B) a disk stack centrifuge operated at 50 and 250 Lh^{-1} . The lysis procedure was performed at 0.5 (small) and 300 (med.) ml scale. Clarification was carried out by filtration (filt.) or batch centrifugation (cent.). Some small-scale preparations were incubated with alkali and detergent for 1 h before neutralisation. (C) Isopropanol precipitated DNA extracted from host cell containing no plasmid (H). Chr is chromosomal DNA, other abbreviations same as Fig 3.2. Gels were 0.8% run for (A-B), 12 h at 40V gel; (C) 3 hrs at 80V.

Sample	% of chromosomal DNA in total DNA
Qiagen purified -CSA1 (250 L h ⁻¹)	39.1%
Isopropanol precipitated DNA -CSA1 (250 L h ⁻¹)	35.6%
Qiagen purified -CARR (60 L h ⁻¹)	13.4%
Isopropanol precipitated DNA - CARR (60 L h ⁻¹)	1.0%
Qiagen purified-JA10	4.3%
Isopropanol precipitated DNA -JA10	0.0%

Table 4.1: Chromosomal contamination (w/w) as assessed by qPCR for DNA extracted by Qiagen and isopropanol precipitation from cell pastes harvested from pilot plant scale fermentation using the CARR and CSA1 centrifuges at 60 L h⁻¹ and 250 L h⁻¹ respectively. Data supplied by Simyee Kong, University College London.

It should be noted that the agarose gel shown in Figure 4.1 is highly overloaded. This has lead to the drifting of the OC band downwards for the CARR derived DNA. This overloading was utilised because it showed any chromosomal and linear forms of DNA which may have been in too low a concentration to be seen for the CARR and CSA1 samples. Figure 4.2 shows DNA that is still overloaded but at a lower concentration ~200 ng per well and this shows distinct bands of OC and SC for the CARR samples. This presence of both SC and OC for the CARR derived samples is borne out by further analysis of the cell paste.

The effect of inefficient mixing at the neutralisation stage was also examined for material derived from the disk stack and the tubular bowl centrifuges. For such experiments, the cell suspensions were incubated with alkali and detergent for 1 hour before neutralisation in order to mimic the effect of extended high pH exposure on plasmid DNA. DNA precipitated from cell suspensions overexposed to lysis buffer

did not show significant changes in the profile of contaminant species compared to those treated with alkali for ≤ 10 min (Figures 4.2A & B). This result is in agreement with the results reported by Kahn et al., 2000, in which lysates were exposed to high pH during prolonged periods of time and the plasmid product was analysed by agarose gel. Further experiments would be required to determine if other changes, not detectable by agarose gel electrophoresis, occur in samples overexposed to high pH and to establish the significance of such changes within the context a large-scale pharmaceutical process.

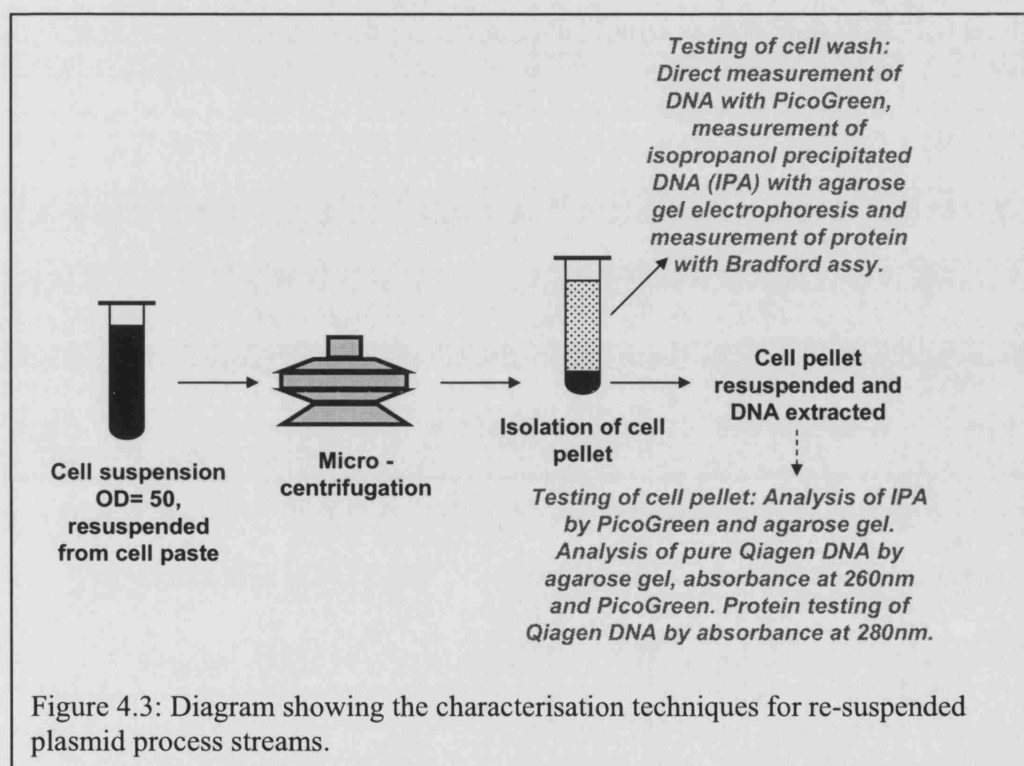
The isopropanol precipitated DNA (IPA) derived from the various operating conditions described above was further purified using mini-chromatographic columns and the DNA obtained loaded onto an agarose gel. Notably, under the conditions used (200 ng in each lane), the chromosomal DNA band observed in samples shown in Figure 4.2B was hardly evident. It is common practice to evaluate cell paste quality and plasmid early recovery strategies by analysis of mini-column purified DNA. The results above indicate that such practice could give insufficient process information and highlight the importance of establishing appropriate process analytical technologies.

4.2.2 Characterisation of cell suspensions

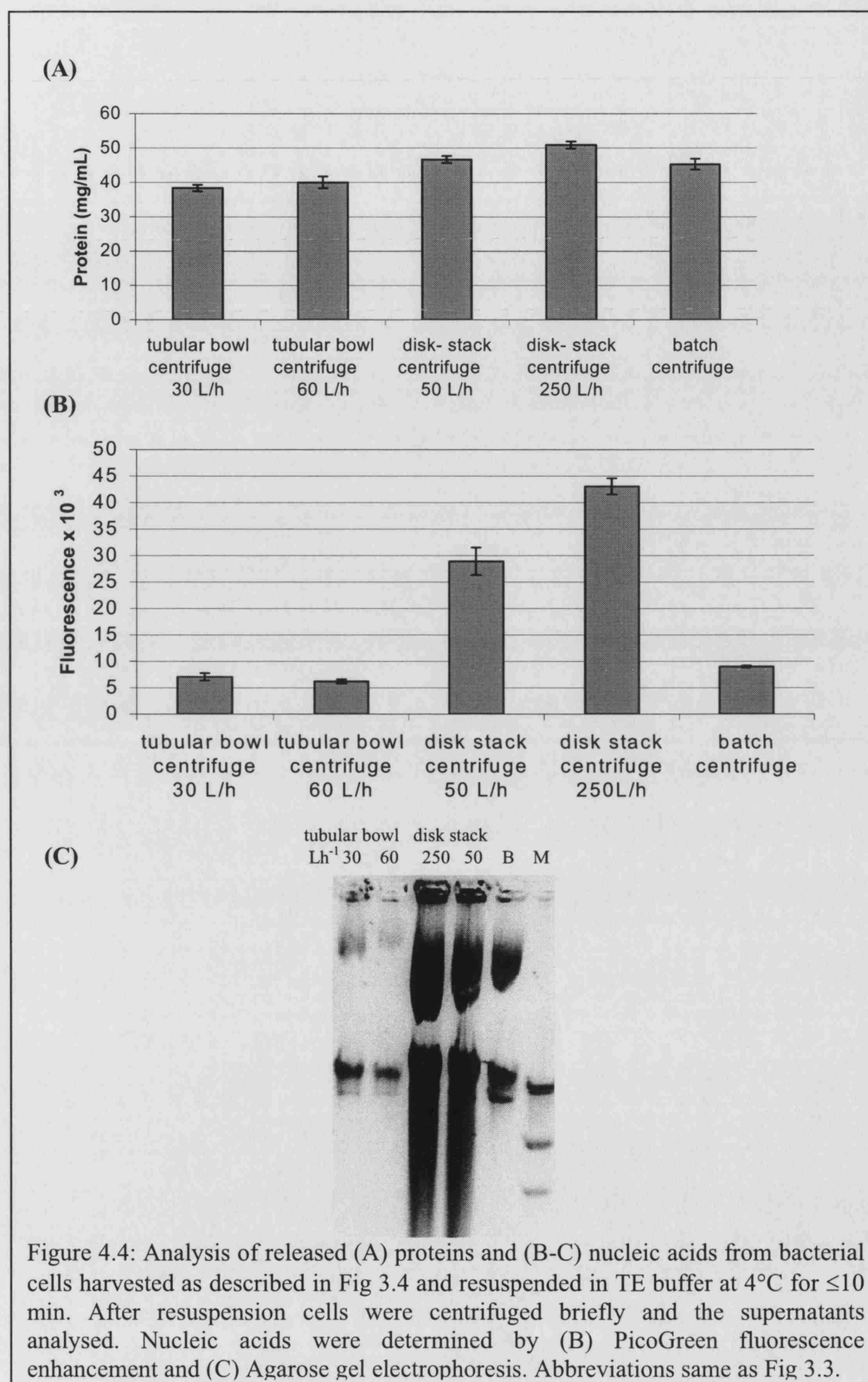
In order to characterise the resuspended cells prior to proceeding to the alkaline lysis stage, three different techniques were examined, as shown in Figure 4.3. Cell suspension was prepared in TE buffer, a small aliquot was taken and spun-down in a microcentrifuge, the supernatant was subjected to protein and DNA analysis.

Analysis of protein content in the supernatant of centrifuged suspensions using the Bradford assay is a well-accepted technique that is insensitive to many of the

substances commonly present in buffers and culture media (Lucarini and Kilikian, 1999). Under the conditions used, protein release from disk stack samples was 12.5-25 % higher than from the tubular bowl (Figure 4.4A). Control samples centrifuged in a batch laboratory centrifuge showed protein release values similar to those of the material harvested at 50 L h^{-1} with the disk stack centrifuge (Figure 4.4A).



Analysis of total nucleic acids released at the cell resuspension stage was carried out by PicoGreen assay. The amount of nucleic acids released from the disk stack centrifuge was 3-4 times higher than from the tubular bowl centrifuge (Figure 4.4B). Control samples centrifuged in a batch laboratory centrifuge showed fluorescence values slightly higher than those from cells harvested with the tubular bowl centrifuge (Figure 4.4B).

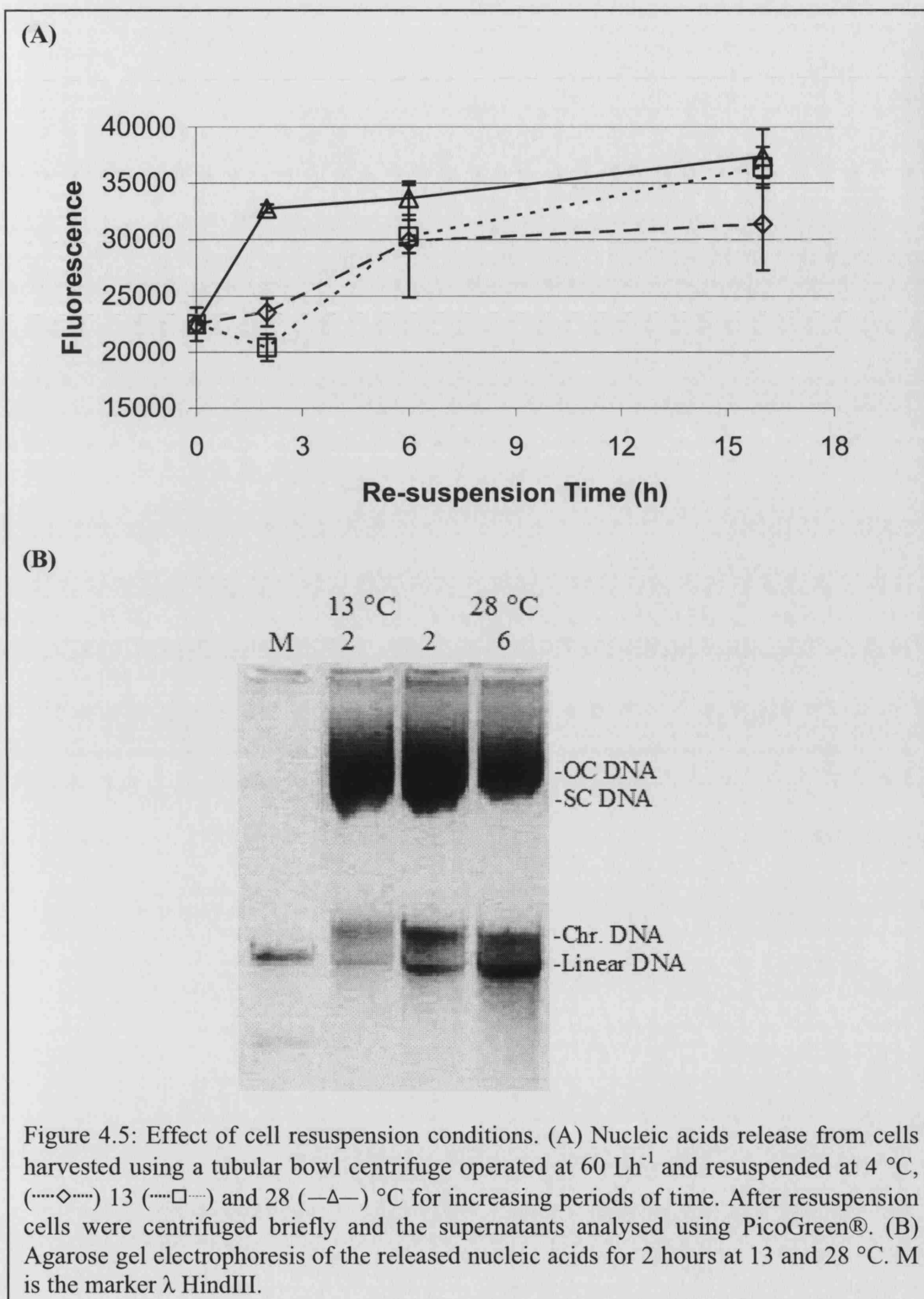


Analysis of total nucleic acid was also carried out by agarose gel electrophoresis in order to identify the species released during resuspension. Equal volumes of supernatants were concentrated by isopropanol precipitation and loaded onto an agarose gel. The samples derived from the disk stack centrifuge showed the highest levels of DNA released, mainly corresponding to chromosomal, open circle and linear plasmid DNA (Figure 4.4C). The batch centrifuge control presented a similar profile but the intensity of bands were much lower. The tubular bowl samples presented a clear band corresponding to chromosomal DNA and only faint bands corresponding to plasmid species (Figure 4.4C).

Taken together, data of Figures 4.4 A-C indicated that results shown in Fig. 4.1 and Fig. 4.2 could be attributed to cell damage associated with the centrifugation operation. The three techniques examined had the same trends but the PicoGreen assay proved to be the most sensitive and rapid of all and was chosen for subsequent experiments. PicoGreen offers a number of advantages over other DNA intercalating dyes and it is increasingly being used for real-time characterisation of process streams (Charlton et al., 1999; Noites et al., 1999; Levy et al., 2000a)

The effect of the cell resuspension operation conditions was also examined. Frozen cells harvested with the tubular bowl centrifuge were thawed, resuspended to $OD_{600} = 65$ in TE buffer and incubated at 4, 13, 28 °C (room temperature) for increasing periods of time, from 0 to 16 h. At given time intervals, a small volume of each of the cell suspensions was taken, spun down and kept at -20 °C for subsequent PicoGreen assay. As shown in Figure 4.5A, for all the 3 temperatures tested there was an increase in nucleic acids release with increasing time. Notably, only 2 hours at room temperature (28 °C) produced a ≥ 45 % increase in fluorescence signal when

compared to the 4 and 13 °C samples, the increased fragmentation of this sample was confirmed through agarose gel electrophoresis as shown in Figure 4.5B. Supernatants derived from suspensions incubated at room temperature alongside a 13 °C control were precipitated with isopropanol and subjected to agarose gel electrophoresis. Results obtained (shown in Figure 4.6) were in agreement with PicoGreen data. Released DNA species corresponded to chromosomal, open circle and linear plasmid forms. It was noted that a proportion of the open circular forms observed after 2 h of incubation were subsequently converted into linear forms (6 h) and further degraded into low molecular weight species after 16 h (Figure 4.6). Agarose gel analysis of isopropanol precipitated DNA extracted from cells subjected to 16 h resuspension at room temperature showed a ≥ 30 % decrease in SC content and a $\geq 10\%$ increase in low molecular weight contamination when compared to a sample resuspended for 2 h at 4 °C.



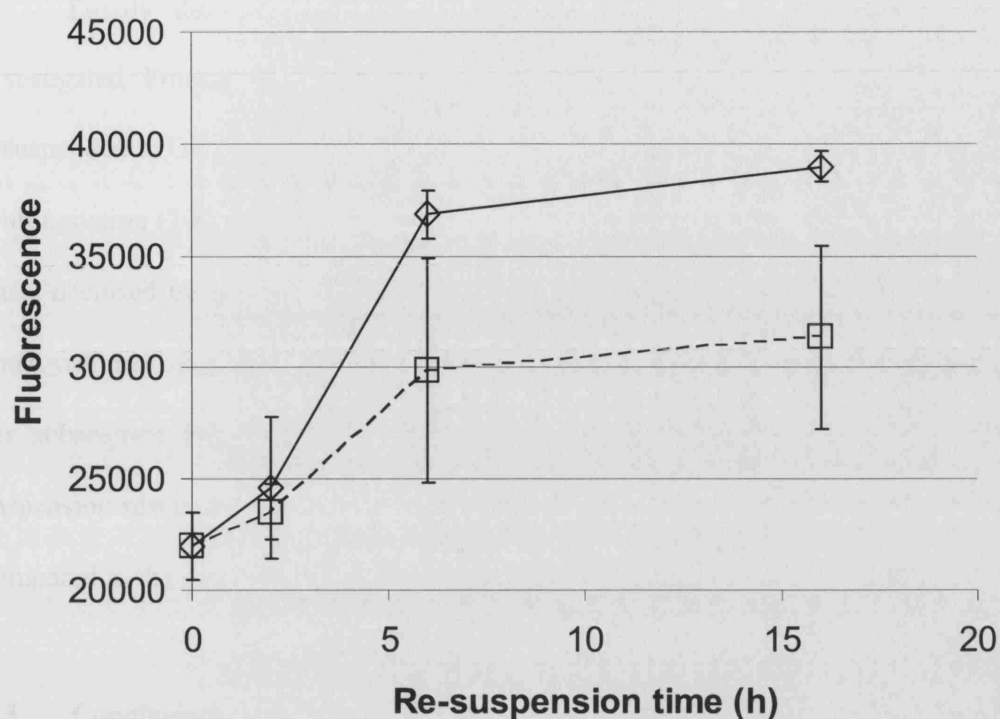


Figure 4.6: Effect of cell resuspension conditions. Nucleic acids release from cells harvested using a tubular bowl centrifuge operated at 60 L h^{-1} and resuspended at 4°C with agitation (—◇—) or under static conditions (---□---) for increasing periods of time. After resuspension cells were centrifuged briefly and the supernatants analysed using PicoGreen.

Plasmid DNA extracted from cells resuspended at 13°C for 0-6 h was purified with mini-chromatographic columns and analysed by qPCR. Electrophoretic analysis showed that the control sample (< 15 min resuspension) had mainly SC plasmid forms, by contrast samples incubated 2-6 h showed increased levels of OC and linear forms. The chromosomal contamination of the purified samples increased with incubation time, yielding values of 3, 14, 10 % w/w for 0 (control), 2 and 6 h of resuspension respectively (qPCR data was provided by Simyee Kong, University College London).

Finally the effect of agitation during the cell resuspension stage was investigated. Frozen cells harvested with the tubular bowl centrifuge were thawed, resuspended to $OD_{600} = 65$ in TE buffer and incubated for increasing periods of time with agitation (200 rpm) or held (static) at 4 °C whereupon mixing of defrosted cell paste occurred by gentle manual shaking for ten seconds. At given time intervals, a small volume of each of the cell suspensions was taken, spun down and kept at -20 °C for subsequent PicoGreen assay. As shown in Figure 4.6, agitation of the cell suspension resulted in a ≥ 20 % increase in fluorescence signal at 6 and 16 h when compared to the static control.

4.3 Conclusions

Cells grown in a 450 L bioreactor were used to establish the impact of pilot scale centrifugation equipment and resuspension operating conditions in the context of plasmid processing. Results have indicated that cell harvesting and resuspension can strongly affect the product yield (due to conversion of the supercoiled form into OC and linear) and the level of chromosomal contamination of the product stream. It has been demonstrated that most of the degraded DNA generated at the host cell recovery stages could not be removed during the alkaline lysis/flocculation step or with the mini-chromatographic columns used in this study.

It is speculated that cell membrane damage induced by shear combined with enzymatic and chemical degradation occurring at the periplasmic and extracellular space contributed to the results obtained (Figs. 4.1 to 4.6). The level of shear occurring at the discharge region of the disk stack centrifuge can be high enough to damage microbial cells (Gerard Chan, University College London, personal

communication). The shear levels will depend primarily on the rotational speed during discharge and at 250 L h^{-1} , 10% w/w cell lysis is expected. Results shown in Fig 4.1 indicate that a sacrifice in throughput could lead to improved overall performance. By contrast, the high shear fields at the entrance of the tubular bowl centrifuge do not seem to have a detrimental effect in the context of cell harvesting of this intracellular product.

Results reported in Fig 4.5B, show that a substantial proportion of SC plasmid form is converted to OC within 2-6 h of cell resuspension at 13°C , and seems to indicate residual enzymatic activity (Middaugh et al., 1998; Walther et al., 2003). *E. coli* DH5 α , which is the current most popular strain used by academic and industrial groups, has an *endA* point mutation that renders it nuclease A defective. Despite this point mutation, it has been recently reported that DH5 α cells show traces of endonuclease activity in the periplasmic space, such activity is not detected in deletion mutants (Griffith and Gietz, 2003). Work conducted at University College London has recently developed a number of genetically engineered *E. coli* strains to aid processing (Cooke et al., 2001; Cooke et al., 2003; Ward et al., 2000). A mutant specifically engineered to prevent SC plasmid and chromosomal DNA nicking during cell recovery stages could provide a means for a more robust and consistent process.

In this thesis, a number of techniques to characterise the cell slurry before proceeding to the alkaline lysis stage were evaluated. It was found that the amount of released nucleic acids at the resuspension stage was inversely proportional to product yield and directly proportional to the level of chromosomal contamination of purified samples.

Direct measurement of released DNA at the cell resuspension stage can provide a simple and fast (~ 10 min) process analytical technology to evaluate product quality before proceeding further downstream. Such evaluation is critical in the context of pharmaceutical processing since fragments of chromosomal DNA that are not entrapped in the cell debris flocculent and remain in solution are likely to co-purify with plasmid DNA, increase fouling and/or significantly reduce chromatography column capacity (Schmidt et al., 1999). Likewise, plasmid DNA that is converted to OC and linear forms will be difficult to separate from the desired SC product and will use the limited capacity of the chromatographic media.

The need to further characterise cell paste leads to the work where the plasmid DNA product itself is analysed, see Chapter 5.

5 CHARACTERISATION OF PURE DNA SOLUTIONS USING A MICROPLATE BASED ASSAY

5.1 Introduction

The increasing use of high molecular weight DNA vectors, such as bacterial and yeast artificial chromosomes for gene expression and gene therapy protocols, requires the development of sensitive analytical tools to monitor DNA quality and consistency during processing and storage (Butler, 1996; Wade-Martins et al., 1999; Campeau et al., 2001; Compton et al., 1999).

Additionally, transfection efficiency of mammalian cells in culture has been shown to be dependent on DNA integrity (Cherng et al., 1999; Xie et al., 1992; Weintraub et al., 1996), whereby the condensed supercoiled plasmid form is the preferred topological form. This is due to the greater transfection efficiency and protection against cellular enzymatic degradation. Regulatory requirements also deem that plasmid forms should show greater than 95% supercoiled formation. Thus, new methods to quickly assess the formation, and thus potency, of DNA solutions are desirable.

Traditional methods for assessing integrity of high molecular weight DNA include alkaline sucrose gradient centrifugation (Bermudez et al., 1999; Brambilla et al., 2000) and filter-binding alkaline elution assays (Guan et al., 2000). Methods to detect apurinic sites usually involve 30 minutes of enzymatic digestion followed by agarose gel electrophoresis that may take from 0.5 to 24 h to run.

Recently, two papers have been published that take advantage of the high sensitivity and specificity of the PicoGreen fluorochrome (Singer et al., 1996) for dsDNA to develop methods to measure DNA integrity (Levy et al., 2000a; Batel et al., 1999). In 2000, Levy et al. reported a method based on the reversible denaturation

properties of closed covalent circular molecules subjected to a heat (95 °C) and rapid cool treatment. Since reversible denaturation conditions for vectors beyond 20 kb could prove to be cumbersome to establish, the use of this method is, in practical terms, limited to solutions containing low molecular weight vectors. In 1999, Batel et al. reported the use of PicoGreen under alkaline conditions to measure the rate of chromosomal DNA unwinding in cells and tissues that had been subjected to radiation and nitroquinoline treatment. Their assay, which is dependent on the number of cells per well, involves a 40 minute lysis treatment in the presence of urea, SDS and EDTA followed by time-course evaluation of the extent of denaturation for less than 1 h.

Thus, a method for detecting single stranded DNA breaks in plasmids and bacterial artificial chromosomes in solution that overcame some of the limitations of previously described methods was sought. The method developed, which takes advantage on the high stability of PicoGreen under alkaline conditions reported previously by Batel et al., 1999, is precise, rapid and the results are independent of the amount of DNA in the well.

Under controlled alkaline conditions, the degree of denaturation of dsDNA molecules is known to increase with an increasing number of breaks and alkaline labile sites, *i.e.* depurinated sites (Lindhal and Anderson, 1972; Drinkwater et al., 1980). Purine bases may be released from DNA in acidic solutions, although depurination also occurs at a physiologically significant rate at neutral pH (Lindhal and Nyberg, 1972). The DNA chain is susceptible to alkaline hydrolysis at these depurinated sites leading to chain breakage. This occurs preferentially at neutral pH and is exacerbated in the presence of Mg^{2+} ions, primary amines and high concentrations of basic proteins (Tamm et al., 1953; McDonald and Kaufmann,

1954). As unwinding begins independently at each break in the chain, the DNA remaining, after the addition of alkali, is proportional to the molecular weight of the dsDNA between these breaks. In the developed method, fluorescence enhancement of PicoGreen at pH 12.4 is normalised by its value at pH 8 giving a ratio that is proportional to the average number of single stranded breaks per DNA molecule in the sample and, therefore, it is indicative of the topological form of plasmid DNA in the process stream.

A paper concerned with this chapter has been published (Rock et al., 2003), a copy of which can be found in Appendix 1.

5.2 Results

5.2.1 Development of an Assay to Assess Integrity of dsDNA Backbone

5.2.1.1 Assay Introduction

In general, the characterisation of process streams is critical for the design and development of pharmaceutical processes. Subsequent validation, quality control and regulatory approval also require assays that can judge the identity, purity, potency and stability of the active product. Characteristics of successful assays are accuracy, sensitivity, reliability, robustness, reproducibility as well as the ability to easily document the assay results. Although prior research at UCL has focused on the processing and monitoring of plasmid vectors of 7-30 kb in size, there are strong indications that the size of vectors used in gene therapy and vaccines will increase to achieve more effective therapy and immune protection.

Compared to smaller plasmids those in excess of 20 kb tend to have low copy numbers. This results in low yields and higher contamination of the plasmid product by chromosomal DNA, the product is also extremely shear sensitive. Current methods

of large plasmid purification and monitoring are not amenable to industry, they are time consuming, labour intensive and not suitable for automation. Thus, attention was focused on the development of an assay that could be utilised at low concentrations, with a high molecular weight plasmid. The resulting assay arose from the investigation of the behaviour of purified pSV β 6.9 kb plasmid DNA and pQR150 (20kb plasmid) with varying degrees of nicking in the presence of alkali. This assay judges the condition of DNA in solution using a highly sensitive DNA binding dye. The assay developed measures single stranded breaks in DNA by assessing changes in the rate of denaturation by alkali for a fixed time.

5.2.1.2 Assay Development

The standard protocol for quantitation of dsDNA with PicoGreen (Singer et al, 1997) requires resuspension of the DNA sample and dilution of the fluorescent dye with TE at pH 8. Under such conditions, dsDNA molecules are expected to remain double stranded regardless of any single stranded breaks or alkaline labile sites that they may contain. By contrast, at pH greater than 8, denaturation (unwinding) begins independently at each break, the proportion of dsDNA remaining after a given period of exposure to alkali is assumed to be proportional to the average molecular weight between breaks (Rydberg, 1975).

DNA placed in a water bath at 60 °C suffers single strand breakage (Evans et al, 1999); this knowledge was used to obtain samples of plasmid DNA with varying amounts of single strand breakage. DNA treated in the water bath for 24 and 44 hours and control DNA were then tested at varying pHs. When combined with PicoGreen, the fluorescence enhancement of DNA was found to decrease with the addition of NaOH and the decrease was larger with the more damaged DNA. The significance of

the difference in decline between the samples proves that fluorescence decline is due to the structure of DNA and not interaction between NaOH and PicoGreen. Thus, the PicoGreen dye could be used for quantitation of dsDNA content in TE buffer at pH greater than 8. Fluorescence enhancement of pSV β control and chemically degraded samples at increasing pH (pH range 8 to 13) is shown in Figure 5.1.

Above pH 12.8, fluorescence values converged to a value just above background level. This was observed in all experiments and was thought to be due to the complete denaturation of the samples and / or inactivation of the dye at such high pH. Under standard conditions (pH 8), for the same DNA concentration, the control sample gave a lower fluorescence signal than the chemically degraded samples. This is in agreement with previous findings that indicate that fluorescence enhancement of PicoGreen is dependent on DNA tertiary structure (Singer et al, 1997; Levy et al, 2000a).

To allow comparison with the control, fluorescence values obtained at pH greater than 8 were normalised by dividing them by the respective values obtained at pH 8 and the data plotted as relative fluorescence against pH, as shown in Fig. 5.1B. Different denaturation patterns were observed for the control and chemically degraded samples, the differences being greater between pH 9 and 12.4. It was found that control DNA was more resistant to denaturation and that denaturation was progressively more severe with increased length of heat treatment. This indicates a good basis for an assay, as the ratio is sensitive enough for detecting nicks and the decrease is due to changes in structure only. A pH of 12.4 was chosen for all subsequent experiments and became the pH condition for the assay because of the robustness of response to minor variations in pH used in the protocol (Fig.5.1B, inset).

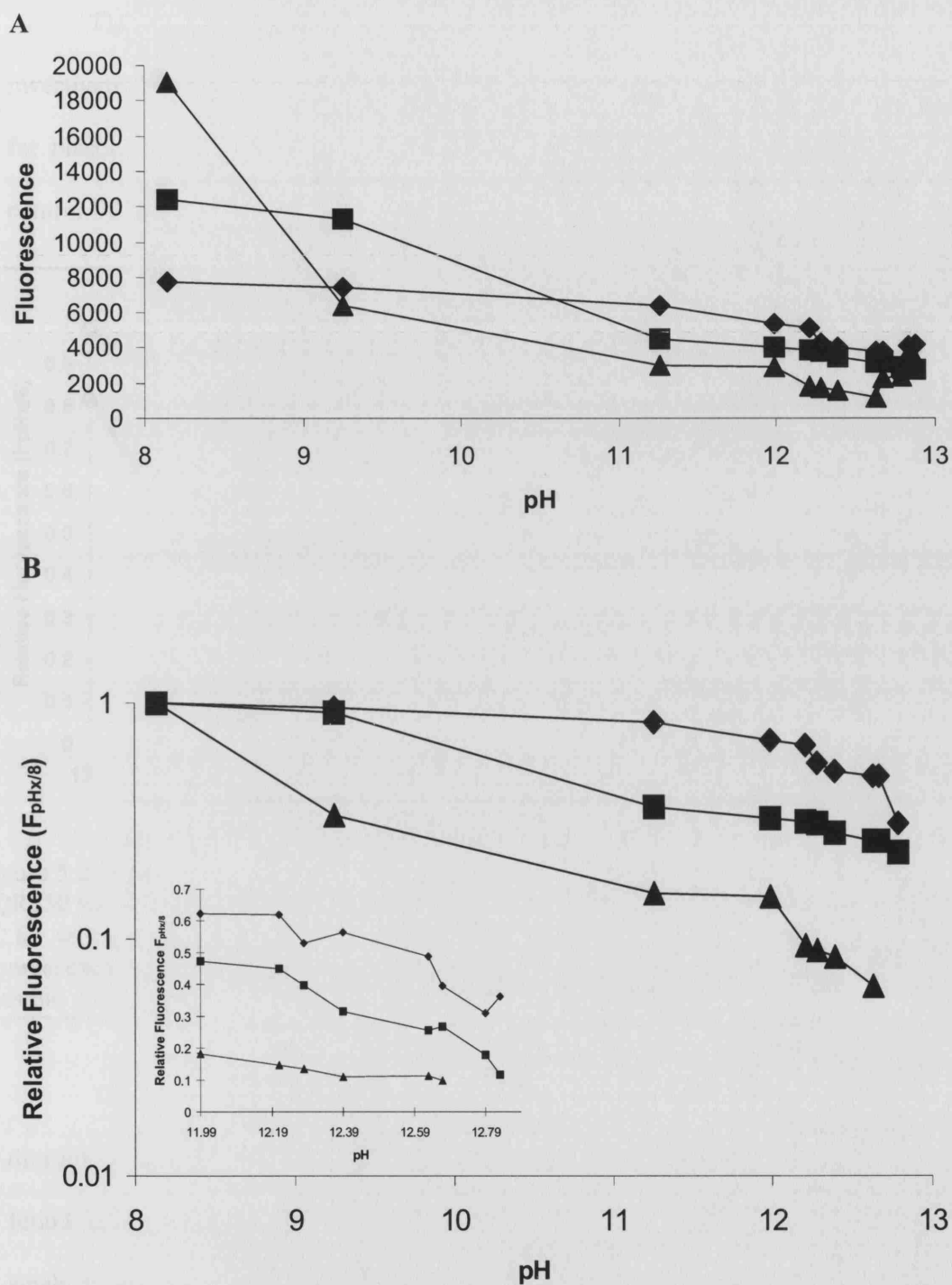
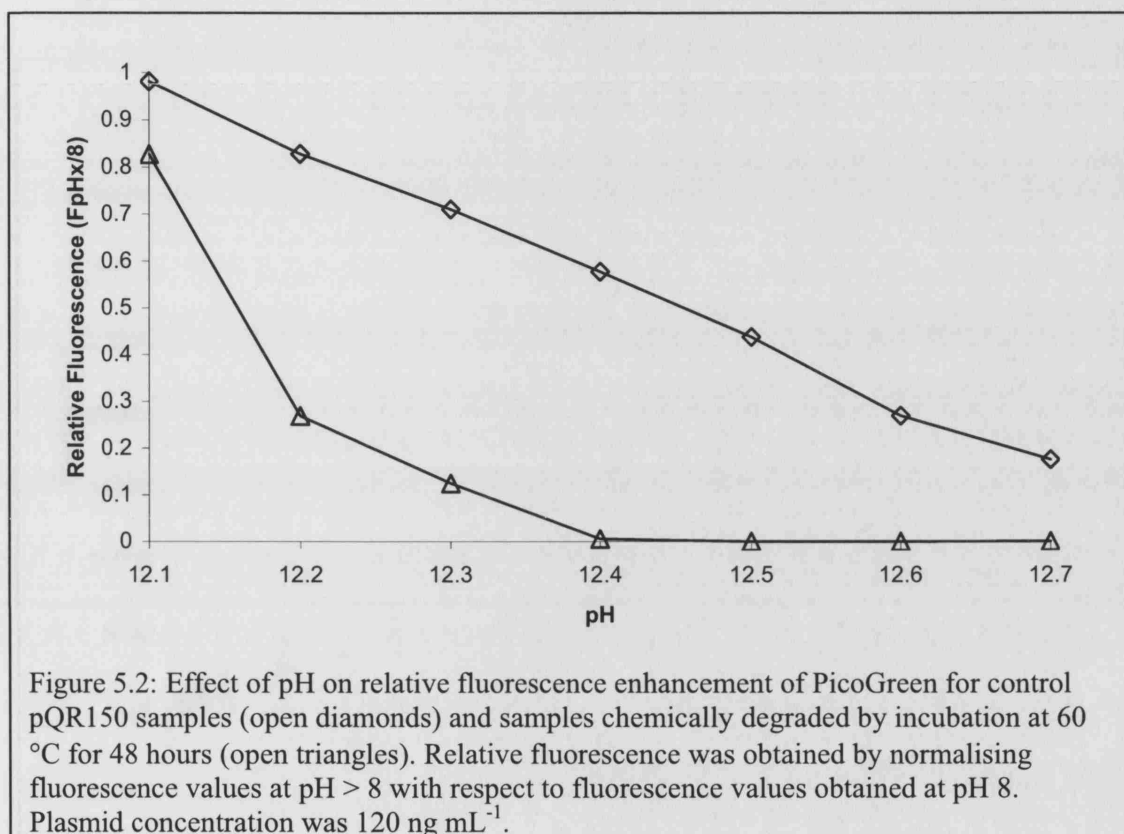


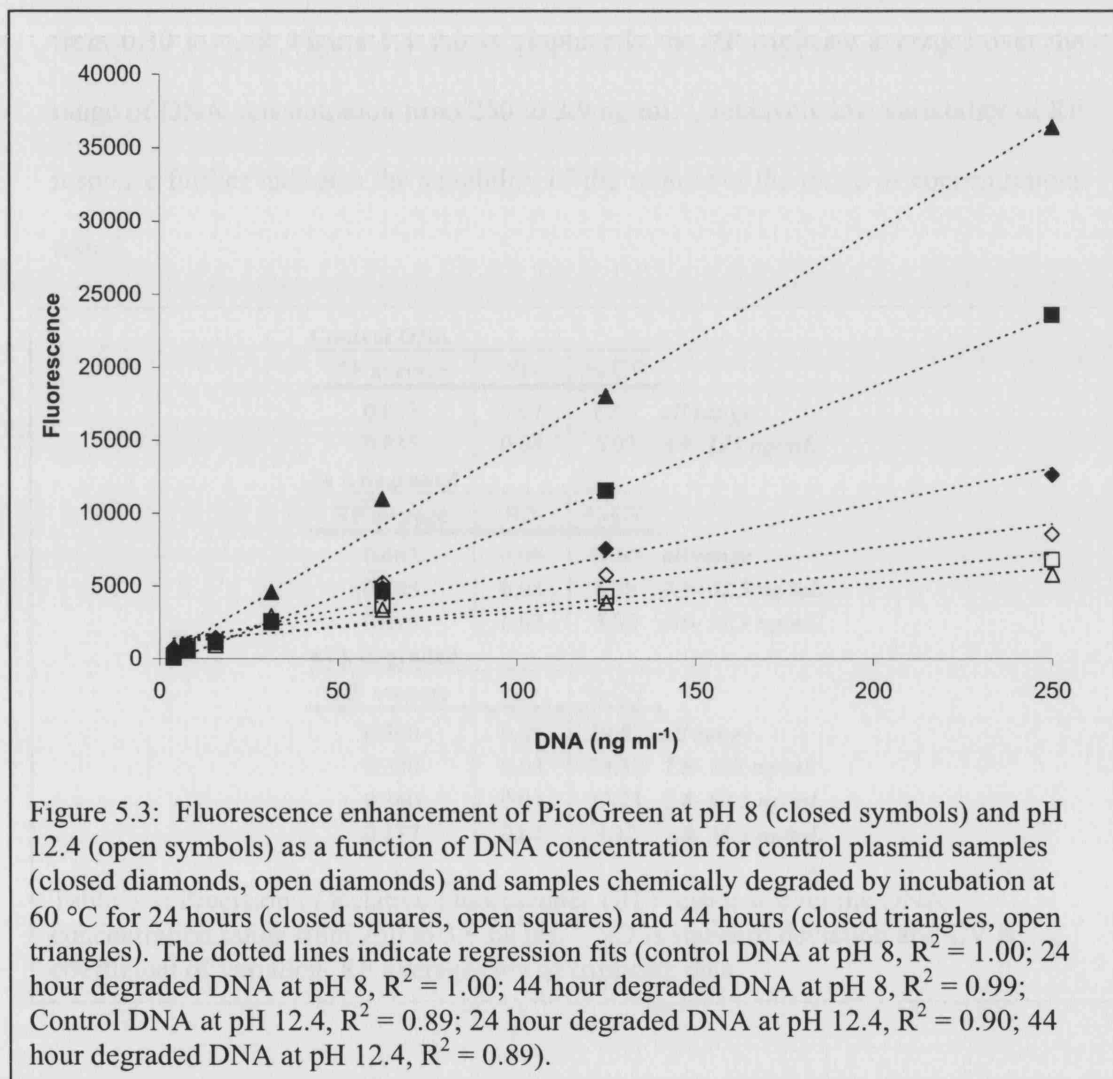
Figure 5.1: Effect of pH on (A) absolute and (B) relative fluorescence enhancement of PicoGreen for control pSV β samples (closed diamonds) and samples chemically degraded by incubation at 60 °C for 24 hours (closed squares) and 44 hours (closed triangles). (B-inset) Magnification of graph B for the region between pH 12 and 12.8. Relative fluorescence was obtained by normalising fluorescence values at pH > 8 with respect to fluorescence values obtained at pH 8. Plasmid concentration was 150 ng mL⁻¹.

The response of purified solutions of the 20 kb plasmid, pQR150, was also investigated with variation in pH. Figure 5.2 shows the differing denaturation patterns for plasmid subjected to heat treatment for 48 hours and control plasmid. Again, control DNA was more resistant to denaturation.



The linearity of 6.9 kb plasmid DNA response was then investigated by serial dilution of control and degraded samples from 250 to 31 ng mL⁻¹. Fluorescence was found to be greatest for 44 hour degraded DNA, followed by 24 hour degraded, and smallest for control DNA. Results shown in Figure 5.3 indicate that there was direct proportionality between DNA concentration and fluorescence with a correlation coefficient of $R^2 \geq 0.89$ at pH 12.4 for the 3 different samples tested. The decrease in fluorescence after the addition of NaOH was greater for a treatment of 44 h than for 24 h, and both were greater than control DNA. Using the data shown in Figure 5.3,

relative fluorescence (RF), the ratio of fluorescence at pH 12.4 and fluorescence at pH 8, was calculated. Results indicated that RF is independent of amount of the DNA in the well, within the range studied. The resulting ratios were in line with previous results: ~ 0.1 - 0.2 for 48 h; 0.27 - 0.5 for 24 h; 0.6 and above for control DNA.



5.2.1.3 Investigation of Assay Precision

Assay precision over the range of DNA concentration from 250 to 3.9 ng mL^{-1} may also be derived from the linearity data. Table 5.1 shows the averages of RF ratios for control, 24 and 44 h degraded 6.9 kb plasmid. The standard deviation and

coefficient of variance of RF values were calculated over the concentration range which was then narrowed in order to pinpoint any areas of increased variability. The plasmid stream with the highest degree of degradation showed the highest variability of RF assay response. Although the coefficient of variation over the full range of DNA concentration was over 29% for 44 h degraded plasmid the average RF ranged from 0.30 to 0.38. Figure 5.4 shows graphically the RF triplicate averages over the range of DNA concentration from 250 to 3.9 ng mL⁻¹, relatively low variability of RF response further indicates the suitability of the ratio over the range of concentrations tested.

Control DNA			
RF average	SD	% CV	
0.813	0.07	8.63	<i>all range</i>
0.835	0.04	5.07	<i>3.9- 125 ng/mL</i>
24 h degraded			
RF average	SD	% CV	
0.463	0.08	17.85	<i>all range</i>
0.508	0.03	6.59	<i>3.9- 62.5 ng/mL</i>
0.495	0.02	3.87	<i>3.9- 31.3 ng/mL</i>
44 h degraded			
RF average	SD	% CV	
0.300	0.09	29.81	<i>all range</i>
0.330	0.08	23.37	<i>7.8- 125 ng/mL</i>
0.360	0.04	12.21	<i>7.8- 62.5 ng/mL</i>
0.377	0.04	9.32	<i>7.8- 31.3 ng/mL</i>

Table 5.1: Precision of Relative Fluorescence (RF) response over the DNA concentration range from 250 to 3.9 ng mL⁻¹. SD is standard deviation and CV is coefficient of variation. RF averages are of triplicate data.

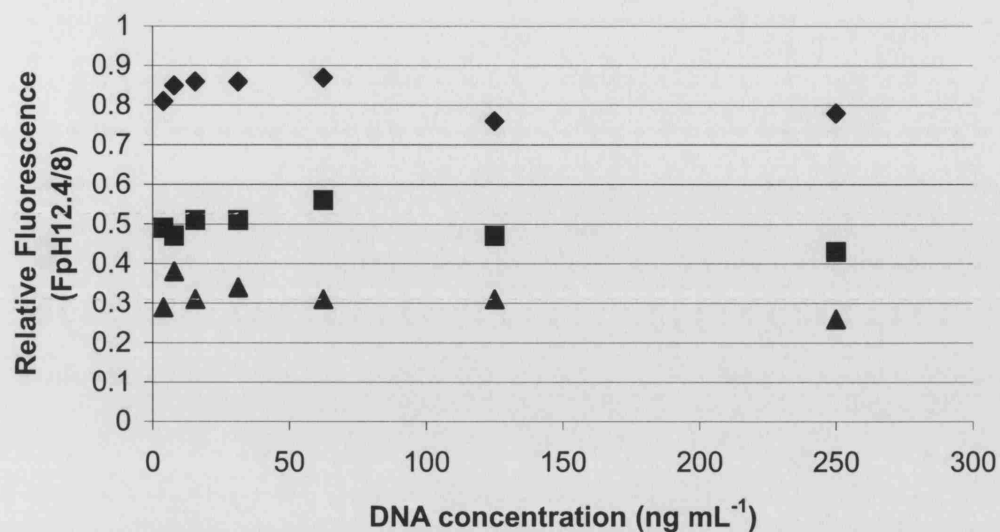


Figure 5.4: Precision of Relative Fluorescence response over a range of 6.9 kb plasmid DNA concentration for control DNA (closed diamonds), 24 h degraded (closed squares) and 44 h degraded (closed triangles).

5.2.1.4 Time-course experiments

The stability of the fluorescence response at pH 12.4 was evaluated with a time-course experiment for pSV β control, 24 and 48 h chemically degraded samples. Following the addition of alkali, fluorescence and RF decreased sharply converging to a steady value after 4 min as shown in Figure 5.5. This agrees with previous observations of the dynamics of DNA unwinding (Rydberg, 1975). Statistical analysis showed that fluorescence values measured between 4 and 7 min remained constant with a 5% coefficient of variation (CV), values measured between 3 and 11 min had a CV of 10%. An incubation time at pH 12.4 of 5 minutes was chosen for the assay and used in all subsequent experiments.

Figure 5.6 shows the Relative Fluorescence (RF) response of control untreated pQR150, 20 kb plasmid, over the course of time. For this plasmid a time of 5 minutes

after the initial drop in fluorescence signal was also found to be suitable, on the same basis as the previous discussion.

Exposure of DNA to high pH has been shown to cause irreversible denaturation (Ciccolini et al., 2002). This seems to be borne out by the decrease in fluorescence signal seen in pH and time course experiments. It is unlikely that this decrease is due to changes in topology as changes from SC to OC and linear DNA has been shown to increase the fluorescence signal as shown in Figures 5.1 and 5.3.

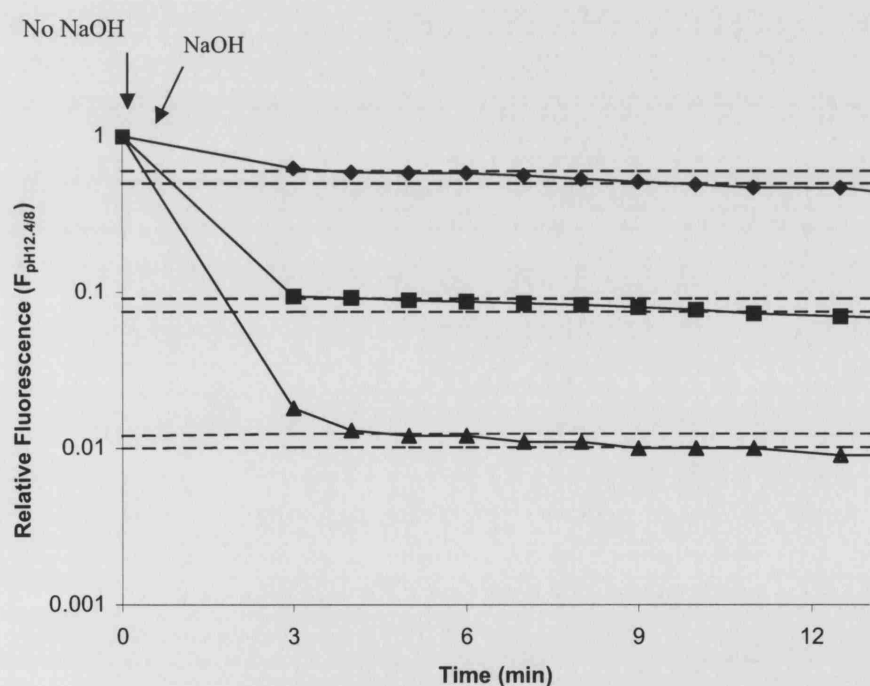
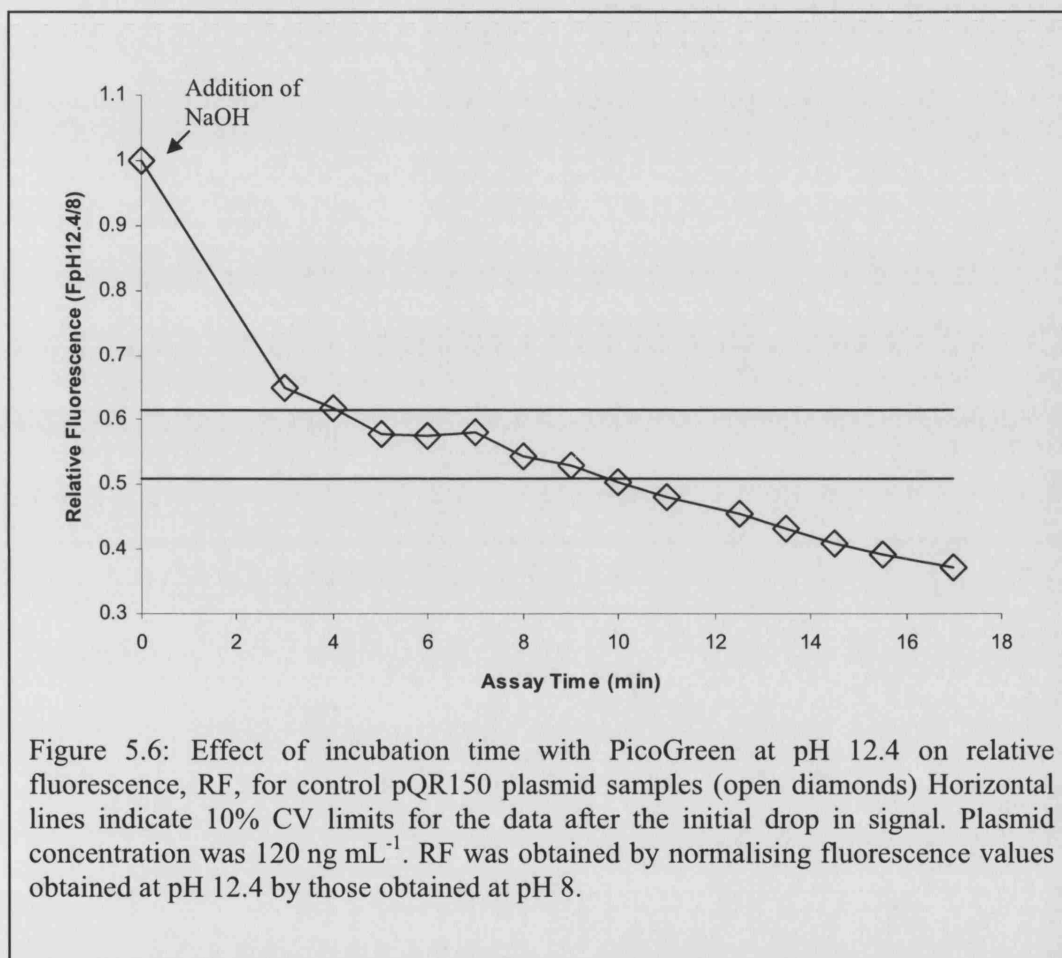
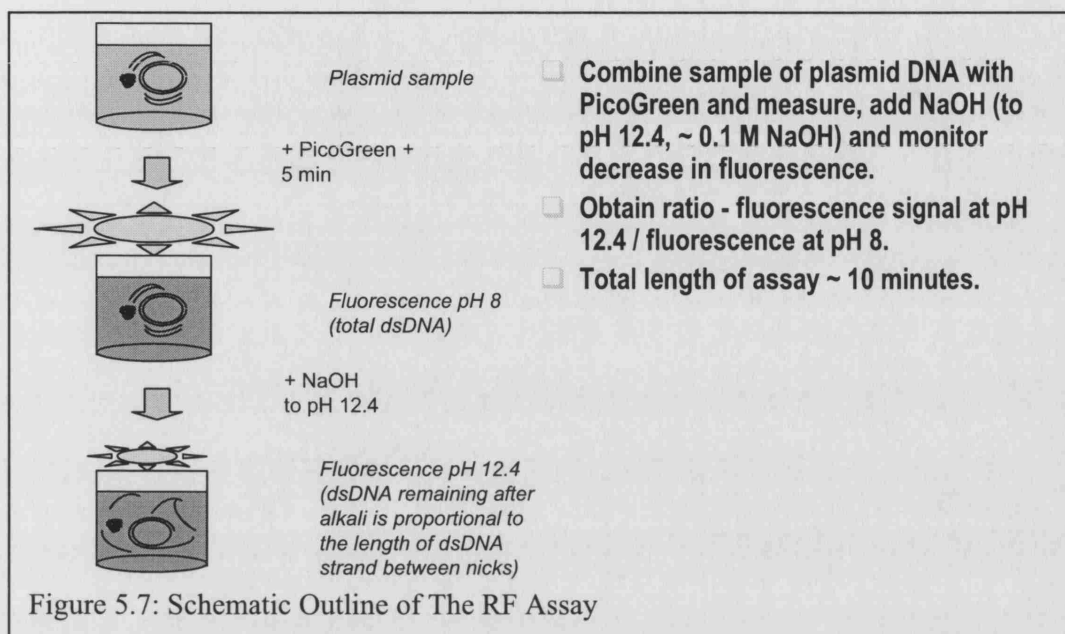


Figure 5.5: Effect of incubation time with PicoGreen at pH 12.4 on relative fluorescence, RF, for control pSV β plasmid samples (closed diamonds) and samples chemically degraded by incubation at 60 °C for 24 hours (closed squares) and 48 hours (closed triangles). Dashed lines indicate 10% CV limits for the data after the initial drop in signal. Plasmid concentration was 320 ng mL⁻¹. RF was obtained by normalising fluorescence values obtained at pH 12.4 by those obtained at pH 8.



5.2.2 The RF Assay

A schematic of the developed assay is shown in Figure 5.7.



5.2.2.1 Assay Validation

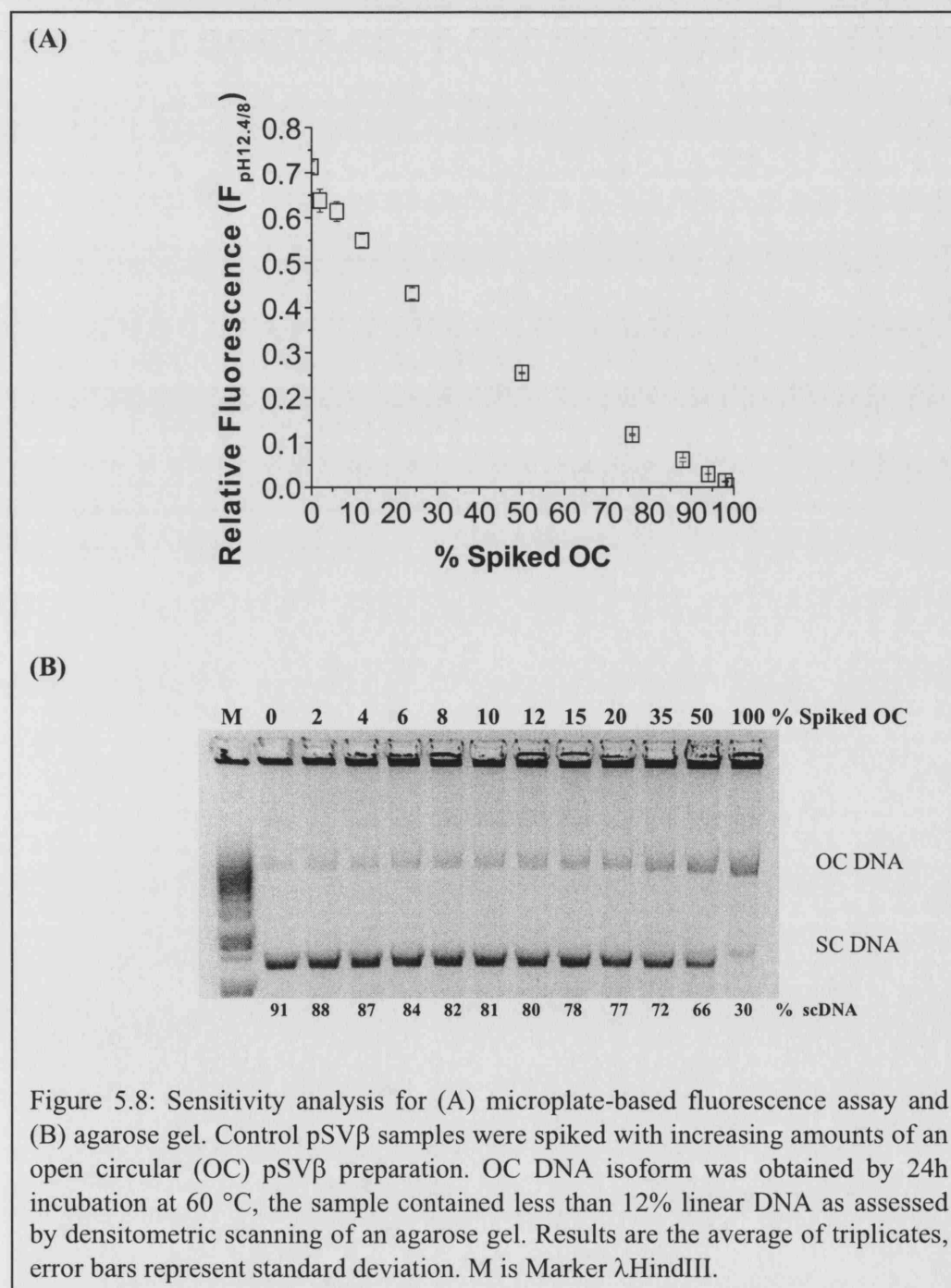
5.2.2.1.1 Sensitivity and Limit of Detection

5.2.2.1.1.1 Theoretical Sensitivity Limits

Data from the RF assay using control and 44 hour degraded DNA from 6.9, 20, 116 and 172 kb plasmids were used to estimate theoretical limits of sensitivity for the assay in each case. Using the values of average and standard deviations for the RF ratios it was determined that the expected limits of sensitivity were 6% degraded DNA for 6.9kb; 12.5% degraded DNA for 20kb plasmid; 12.5% degraded for 116kb BAC and 25% degraded DNA for 172 kb BAC.

5.2.2.1.1.2 Experimental Sensitivity Limits

Sensitivity of the fluorescence assay was experimentally tested by evaluating the lowest percentage of degraded plasmid that could be reliably measured in a sample tested sensitivity of the fluorescence assay. Independent triplicate samples of



degraded and control DNA mixtures, ranging from 100% to 0% control DNA, were assayed and the lowest percentage of degraded DNA at which the signal was statistically different from the control (Student's T-Test, 95% confidence interval) was taken as the limit of sensitivity. For pSV β this limit was found to be 6%. An agarose gel loaded with the same mixtures of pSV β showed no significant difference by densitometric scanning of supercoiled (SC) and open circular (OC) and calculation of the ratio of SC/(SC+OC), for samples containing up to 35% degraded DNA, as shown in Figure 5.8. The values for SC DNA were multiplied by a correction factor of 1.36 to account for the differential binding seen with ethidium bromide and an accuracy of 20% was assumed for agarose gel densitometric scans (Projan et al., 1983; Levy et al., 1999).

For further analysis of the sensitivity of the assay enzymatic breakage of plasmid DNA may be used to introduce a controlled and calibrated number of breaks for a particular plasmid sequence. Radioactivity has been used in the past to induce damage but enzymatic means provide a much safer option. Abasic sites which constitute a common form of DNA damage arise from the spontaneous or enzymatic breakage of the N-glycosyl bond and the loss of a nucleotide base. This type of damage may be induced by the use of DNA N-glycosylases which has been used in combination with abasic site assays by Kow and Dare, 2000.

5.2.2.1.2 Assay Precision under manual operation (inter and intra-assay variation)

Intra-assay precision under manual operation was determined by assaying 3 independent samples of pSV β in triplicate, the percentage coefficient of variation (CV) of RF obtained ranged from 2.9 to 4.1 (Table 5.2). For the inter-assay precision,

independent triplicates of three lots of pSV β were assayed separately and compared, the percentage CV ranged from 1.8 to 3.5 (Table 5.3).

Sample ID*	RF (values)	RF ($\bar{x} \pm \sigma$)	Coefficient of Variation (%)
A	0.621	0.635 \pm 0.026	4.1
	0.620		
	0.665		
B	0.705	0.716 \pm 0.020	2.9
	0.703		
	0.739		
C	0.734	0.717 \pm 0.029	4.0
	0.734		
	0.684		

Table 5.2: Intra-assay precision for manual assay

* Three different samples of pSV β were analysed in triplicate in the same 96 well microplate.

Sample ID*	RF (values)	RF ($\bar{x} \pm \sigma$)	Coefficient of Variation (%)
A	0.699	0.713 \pm 0.013	1.8
	0.724		
	0.715		
B	0.594	0.614 \pm 0.022	3.5
	0.636		
	0.611		
C	0.538	0.549 \pm 0.014	2.6
	0.543		
	0.565		

Table 5.3: Inter-assay precision for manual assay

* Three independent assays for (A) control and (B and C) partially degraded pSV β samples were performed.

5.2.2.1.3 Correlation of assay to agarose gel electrophoresis

The application of the assay towards process streams of plasmid DNA was further established through correlation of assay data to that gained from the best-established plasmid characterisation method, which is agarose gel electrophoresis. For chemically degraded samples of plasmid pSV β (0-42 hours), agarose gel electrophoresis relates the progressive formation of open circular and linear forms of plasmid DNA, Figure 5.9A. Densitometric analysis of the agarose gel was correlated against relative fluorescence of triplicate samples of degraded plasmid. Densitometric data for supercoiled DNA was again multiplied by a correction factor of 1.36 in accordance with Projan et al., 1983. Figure 5.9B shows that there was good correlation ($R^2 > 0.97$) of these samples confirming the sensitivity of the method to changes in plasmid isoform.

Good correlation of densitometric data to RF ratios for pQR150 (20kb plasmid) has also been shown with regression fits were greater than $R^2 = 0.96$.

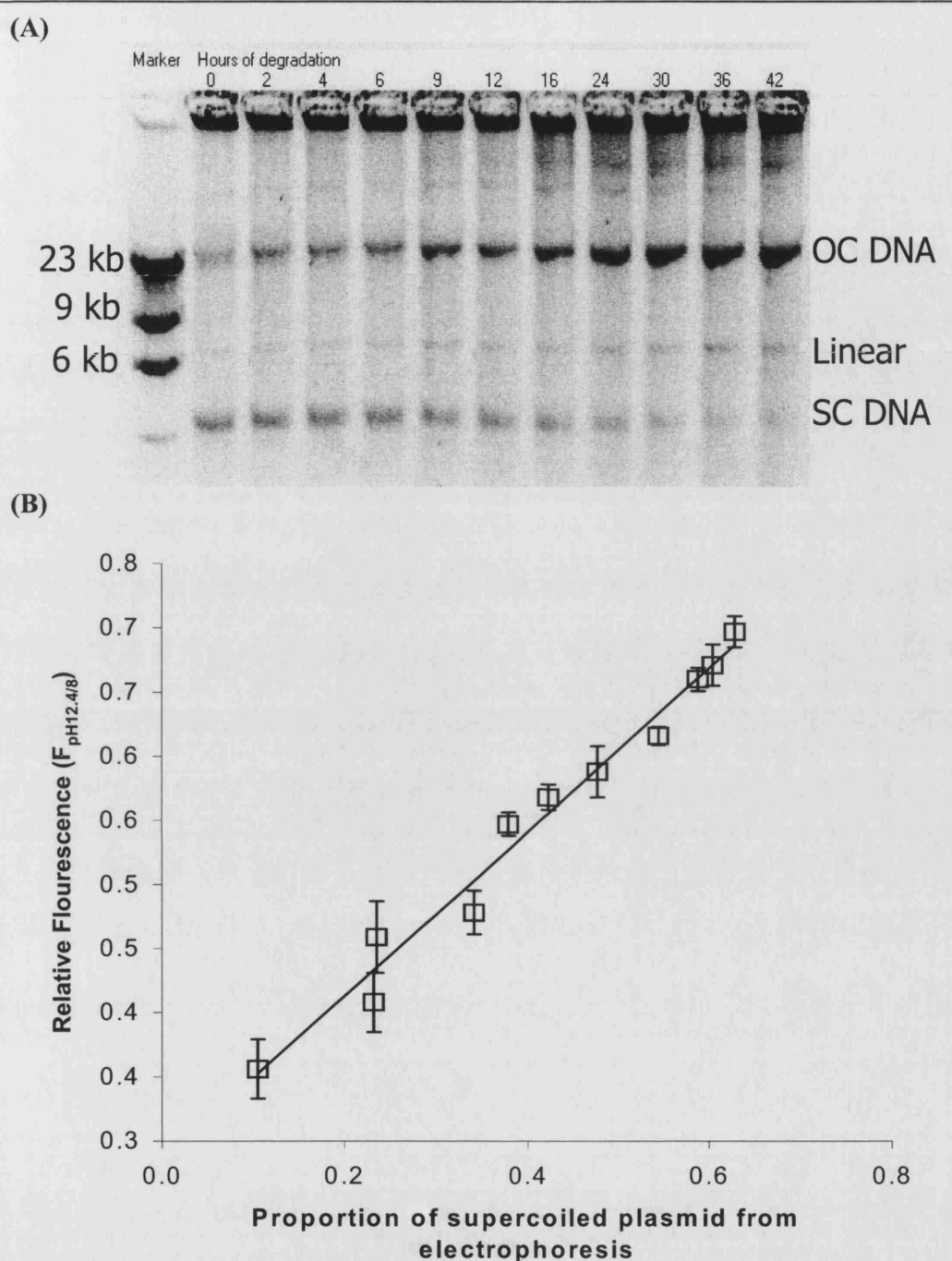


Figure 5.9: (A) Agarose gel electrophoresis of pSV β 6.9kb samples subjected to chemical degradation for 0 to 42 hours. M is the DNA marker λ HindIII. (B) Correlation between relative fluorescence (RF) obtained from microplate-based fluorescence assay and percentage supercoiled plasmid obtained from densitometric scanning of SC, OC and linear DNA. The fluorescence results were the average of triplicate values, error bars represent the standard deviation. RF was obtained by normalising fluorescence values obtained at pH 12.4 by those obtained at pH 8. Solid line, linear regression gave, $y = 0.605x + 0.302$, $R^2 = 0.977$. DNA concentration in microplate based fluorescence assay was 270 ng mL^{-1} .

5.2.2.1.4 Test of calculation protocol

At a fixed pH, the fluorescence of a sample mixed with PicoGreen is directly proportional to the mass of the dsDNA in the sample (Molecular Probes, Leiden). Thus at pH 8:

$$F_{pH8} \propto dsDNA_{pH8} \quad (5.1)$$

where F_{pH8} , is fluorescence of the DNA sample at pH 8, $dsDNA_{pH8}$ represents the total mass of dsDNA at pH 8. Treatment with alkali at pH 12.4 causes the independent unwinding at both ends of single stranded breakages in the DNA backbone. From the data shown in Fig. 2, fluorescence at pH 12.4 may be expressed as follows.

$$F_{pH12.4} \propto dsDNA_{pH12.4} \quad (5.2)$$

where $F_{pH12.4}$, is fluorescence of the DNA sample at pH 12.4.

Combining equations (5.1) and (5.2), the ratio of fluorescence (RF) obtained at pH 12.4 and pH 8 may be expressed as:

$$RF \propto \frac{dsDNA_{pH12.4}}{dsDNA_{pH8}} \quad (5.3)$$

Previous work based on mathematical models of strand separation of irradiated DNA in alkali (Rydberg, 1975; Sheridan and Huang, 1977), has shown that the ratio on the left hand side of equation 5.3 is related to the number of strand breaks and alkaline labile sites (SB) by the following equation.

$$-\ln RF \propto SB \quad (5.4)$$

An estimation of average strand breaks (SB) per plasmid molecule in a population can be derived from densitometric scanning of a gel using the following equation (Evans et al, 1999; Drinkwater et al., 1980):

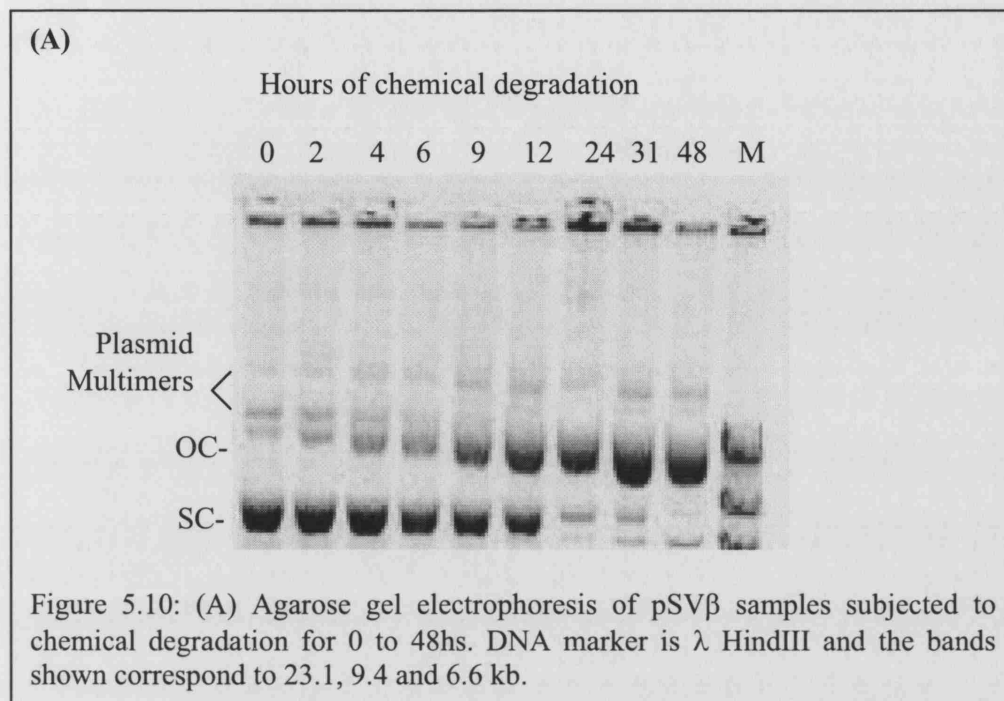
$$SB(gel) = -\ln\left(\frac{SC}{SC + OC}\right) \quad (5.5)$$

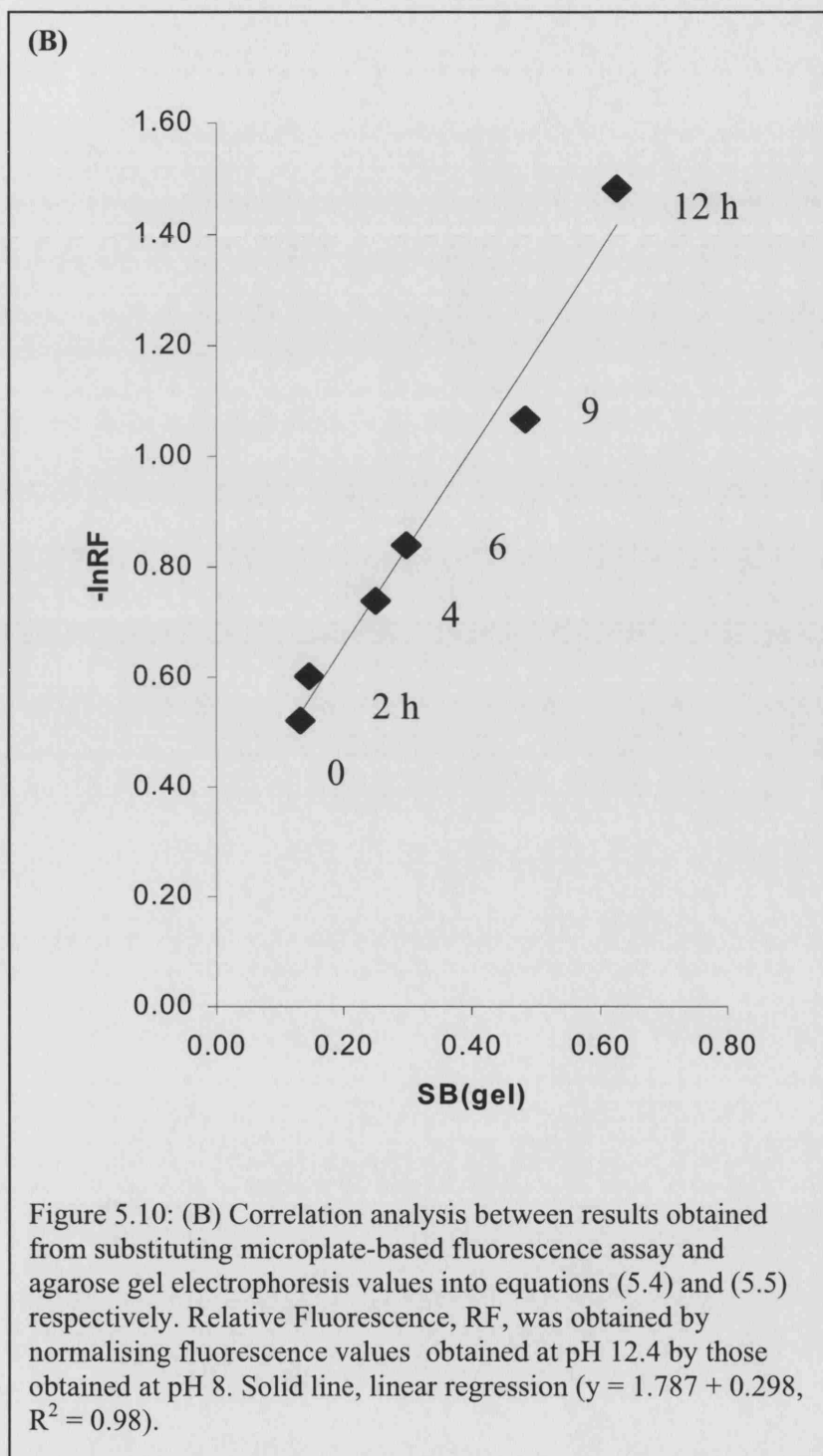
where SC is supercoiled DNA and OC is the open circular form. This estimation is only valid for a dsDNA population that has an average of less than 2 breaks per molecule and it is assumed that any apurinic sites are immediately converted to strand breaks (Drinkwater et al., 1980).

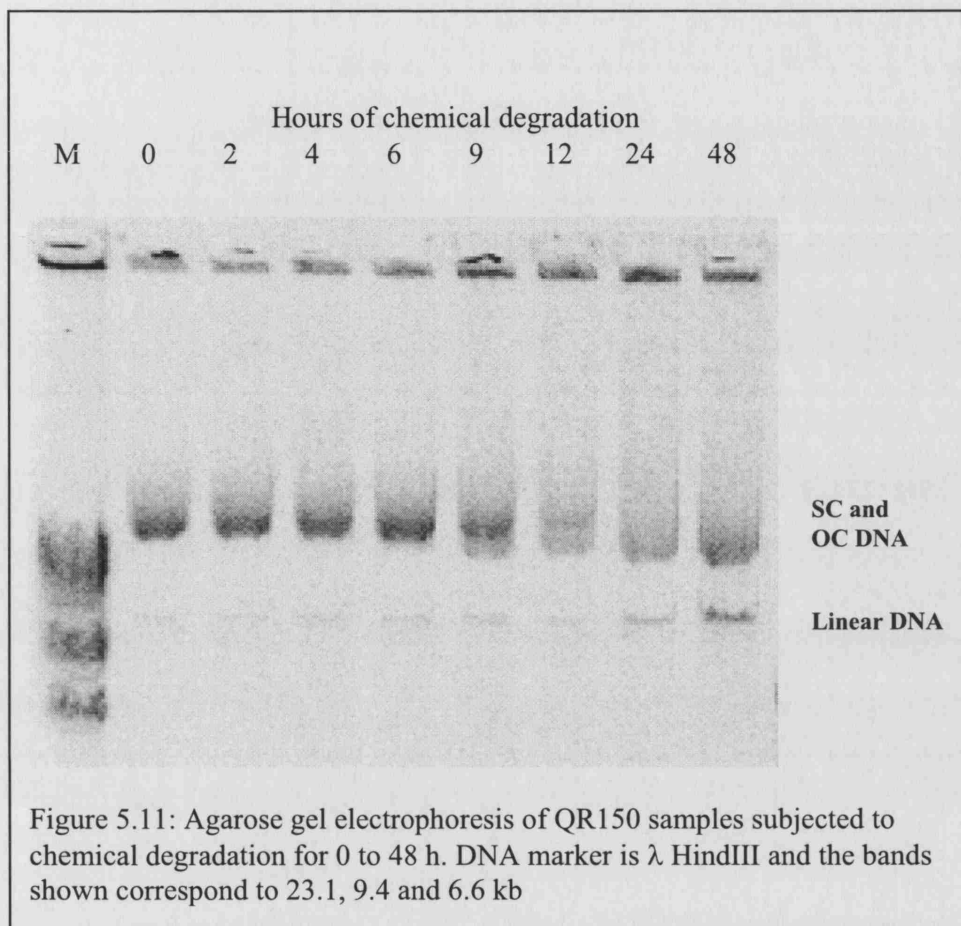
To test the use of equation (5.4) a correlation plot was constructed between values derived from the microplate-based fluorescence assay and those derived from gel electrophoresis analysis. Samples of pSV β subjected to increasing time (0-48 h) of chemical degradation were analysed. Densitometric scanning of the gel revealed that after 12 hours of chemical degradation a proportion of the OC molecules had been converted into linear DNA (Fig. 5.10A), at 12 and 24 hours of chemical degradation the sample was 53 and 24 % SC DNA respectively. In order to ensure that samples analysed had an average of less than 2 strand breaks per molecule, only results obtained from SC and OC scanning between 0 to 12 h were used to construct the correlation plot shown in Fig 5.10B. A correction factor of 1.36 has been used to account for decreased binding of ethidium bromide to supercoiled forms in accordance with Projan et al., 1983. As shown in Fig 5.10B, a good regression fit ($R^2 = 0.98$) was obtained from plotting SB(gel) values derived from densitometric scanning of the gel against $-\ln(RF)$ values calculated from the microplate-based fluorescence assay. As the gradient of the trend line is almost 1, it is indicated that both methods are equally responsive to degraded DNA.

The test of the derived equation 5.4 was successful, however, it is limited by the fact that the linear form of DNA is not taken into account by published equations. Experimentally, correlations between agarose gel electrophoresis and PicoGreen data have shown good regression fits ($R^2 > 0.96$) for samples of 6.9 and 20 kb plasmids

degraded between 0-48 h. Agarose gel electrophoresis for the 20 kb plasmid is shown in Figure 5.11.







5.2.3 Application of the assay to BAC DNA

A preparation of p5176 was incubated at 60 °C to accelerate chemical degradation and samples were taken at regular intervals and assayed, as described in Methods and Materials. Plasmid pSV β was subjected to the same treatment and assayed in parallel as a control (Figure 5.8). When RF values for both p5176 and pSV β at increasing time were normalised by the control RF at time = 0, the normalised values decreased sharply with time between 0 and 10 hours, but decreased at a slower rate after this initial period (Figure 5.12).

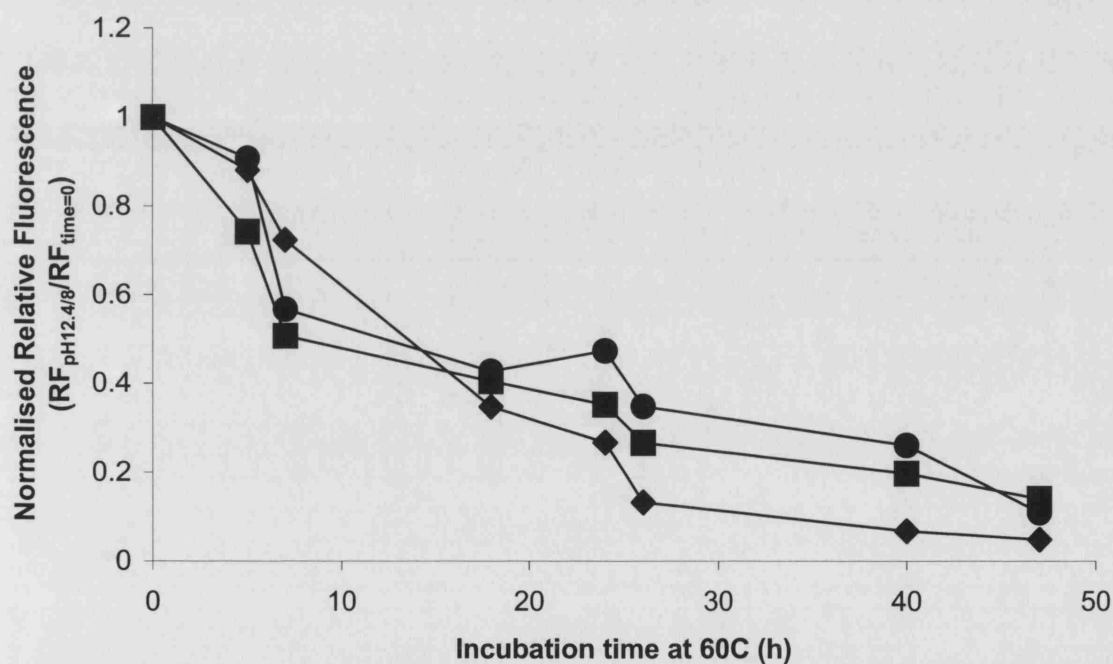


Figure 5.12: Effect of incubation time at 60 °C on relative fluorescence, RF, for pSV β (closed circles), pQR150 (closed diamonds) and p5176 (closed squares). RF values were normalised with respect to values at time = 0. DNA concentration was 300 ng mL⁻¹.

Samples of p5176 were subjected to shear at average rates of 1.6×10^4 - 3.3×10^5 s⁻¹ using equipment and methods described in Chapter 2, and in Levy et al., 1999 and Levy et al., 2000. A sample of pSV β was treated in parallel as a

control. The sheared samples were then assayed as described in the previous section and for direct comparison RF values for sheared samples were normalised by control RF at shear = 0, (Figure 5.13A). The normalised RF for the high molecular weight p5176 decreased sharply at relatively low shear rates $\sim 1 \times 10^3 \text{ s}^{-1}$, indicating that considerable damage to DNA backbone had occurred. As expected, due to its lower molecular weight, pSV β showed no decrease in RF values over the shear rates tested.

A preparation of p5176 was incubated at 60 °C to accelerate chemical degradation and samples were taken at regular intervals and assayed as described in the previous section. Plasmid pSV β was subjected to the same treatment and assayed in parallel as a control. RF values for both p5176 and pSV β decreased sharply with time between 0 and 10 h but decreased at a slower rate after this period.

Samples of p5176 were sheared at average rates of $1.6 \times 10^4 - 3.3 \times 10^5 \text{ s}^{-1}$ using the method of Levy et al., 2000. The plasmid pSV β was sheared in parallel as a control. The sheared samples were then assayed as described in the previous section and RF values compared. As expected, the RF of p5176 decreased sharply at relatively low shear rates $\sim 1 \times 10^3 \text{ s}^{-1}$, in contrast pSV β showed no degradation even at the highest shear rates tested.

A pulsed-field gel electrophoresis of control, chemically and shear degraded 116 and 172 kb BAC samples was carried out. For the 116 kb BAC, the control DNA showed linear and OC bands. The linear form of the plasmid could be seen as a band in a position between the 97 and 146 kb bands of the λ multimer ladder, whereas the OC band, though faint, runs close to the 243 kb marker. With a shear rate of $8 \times 10^4 \text{ s}^{-1}$, the linear band is much more intense and there is no OC band. With 12 h chemical degradation the signal of the linear form is stronger than that of the control though fainter than that of the sheared sample, there is also a faint OC band. These

qualitative results are largely echoed by the 172 kb BAC with the exception of the very decreased linear DNA band for sheared sample, possibly due to the heavy breakage of sample into fragments smaller than 172 kb, Figure 5.13B.

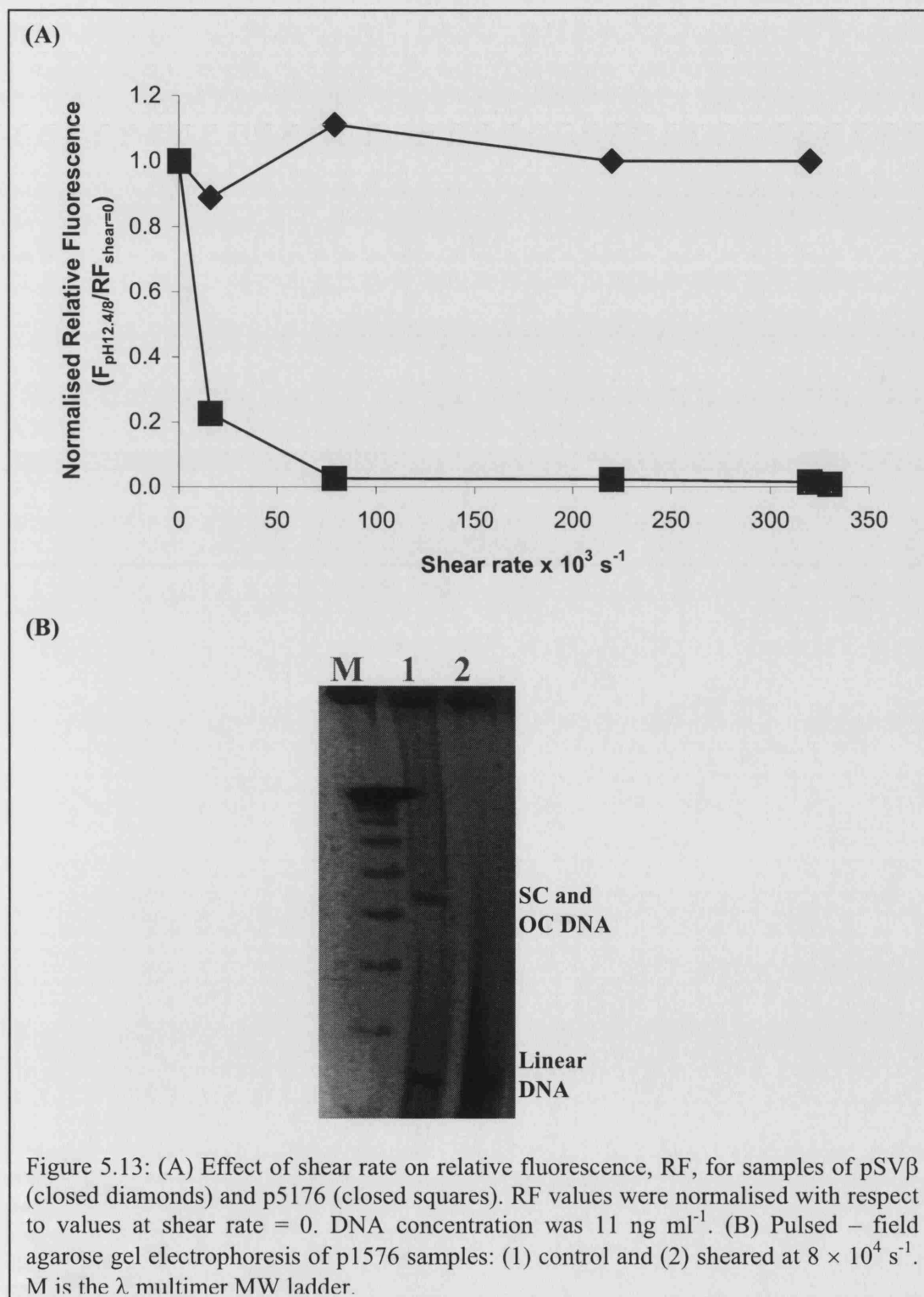
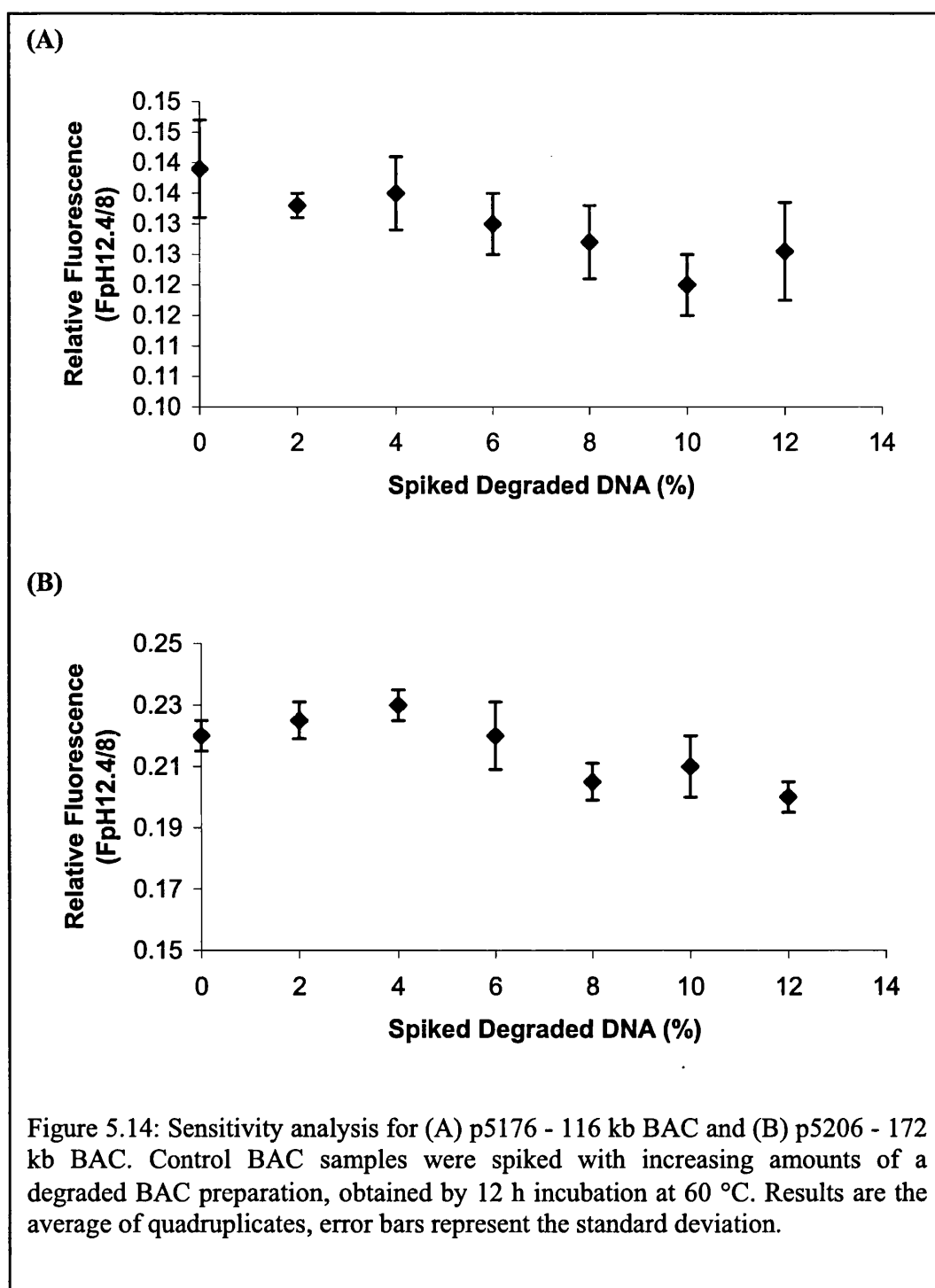


Figure 5.13: (A) Effect of shear rate on relative fluorescence, RF, for samples of pSVβ (closed diamonds) and p5176 (closed squares). RF values were normalised with respect to values at shear rate = 0. DNA concentration was 11 ng ml^{-1} . (B) Pulsed – field agarose gel electrophoresis of p1576 samples: (1) control and (2) sheared at $8 \times 10^4 \text{ s}^{-1}$. M is the λ multimer MW ladder.

Spiking experiments (as conducted for the 6.9kb plasmid) were utilised to confirm the sensitivity of the assay with regard to the 116 and 172 kb BACs. The RF assay was found to be almost as sensitive for the artificial chromosomes as when applied to 6.9 and 20 kb plasmids in the detection of spiked quantities of degraded DNA. As mentioned previously, the theoretical limits of detection for the 116 kb and the 172 kb BAC were 12.5 % and 25 % degraded DNA respectively. Experimentally, the limits for the 116 kb BAC were shown to be 4 % and 10 % at 90 % and 99.5 % confidence limits respectively, as shown in Figure 5.14A. The limit of detection for the 172 kb BAC was found to be 12 % at 99 % confidence limits, Figure 5.14B.



5.4 Conclusions

The microplate-based assay developed provides rapid information on the backbone integrity of dsDNA molecules in solution. The method is simple, takes 10-12 min per 96-well microplate and requires small amounts of sample. The assay is also amenable to high throughput automation and was used successfully to rapidly evaluate chemical and shear-induced damage of an artificial chromosome preparation (Fig. 5.12- 5.13). The method is applicable to a wide range of DNA sequences and sizes and its result is more sensitive than traditionally accepted methods of analysis. Thus, the method provides an attractive option for analysis of backbone integrity of high molecular weight vectors at both laboratory and industrial scale that would otherwise require more time consuming and labour intensive methods. Furthermore, the assay has the potential to detect alkaline labile sites such as apurinic sites and is in principle more sensitive than agarose gel electrophoresis.

The method has been used successfully by other researchers in the Biochemical Engineering Department of University College London to assess the effect of engineering environments within valves, pumps and microfiltration units on the integrity of plasmids from 30-272 kb. The high sensitivity of the PicoGreen allows researchers to use very dilute samples of plasmid DNA (on the order of nanograms of plasmid per mL of solution). This also allows the preparation and efficient analysis of the large volumes required by pilot plant scale equipment. Furthermore, the method was used to evaluate the effect of 0.2 μm sterile membranes on a range of plasmids. Results with the pulsed field electrophoresis gels have demonstrated that the method was particularly appropriate for the detection of backbone alteration in high molecular weight vectors, such as bacterial artificial chromosomes.

6 ANALYSIS OF CELL LYSATES

6.1 Introduction

The ability to reliably predict plasmid yield and quality at any point in the production process before final purification is an invaluable tool for manufacturers. It can immeasurably reduce wasted time, money and energy by enabling the producer to make decisions based on the economic value of the predicted yield. This is quite important at the large-scale where certain steps take long periods of time and can be very expensive. Within this chapter, the development of an assay that can reliably indicate plasmid yield and quality in a mixture as unprocessed and complex as *E. coli* cell lysate was attempted. An assay that could be used on-line during alkaline lysis could be useful to determine the rate of chromosomal DNA denaturation and the overall integrity of chromosomal DNA. Depending on the method used, chemical cell lysate is a mixture of detergents, salts and importantly proteins, RNA, murein and other cellular components. Work by Levy et al., 2000, has demonstrated that the standard PicoGreen assay is sensitive and robust enough to reliably determine plasmid concentration in clarified cell lysates. The investigation focused on the extension of the use of the Relative Fluorescence (RF) assay to crude and clarified lysates. However, the contaminants in this mixture may cause significant interference with any type of fluorescence signal, which may be exacerbated at high pH. Therefore, besides investigating the appropriateness of the assay and any modifications that may have to be made, the trade-offs between the range of assay and its sensitivity were also considered.

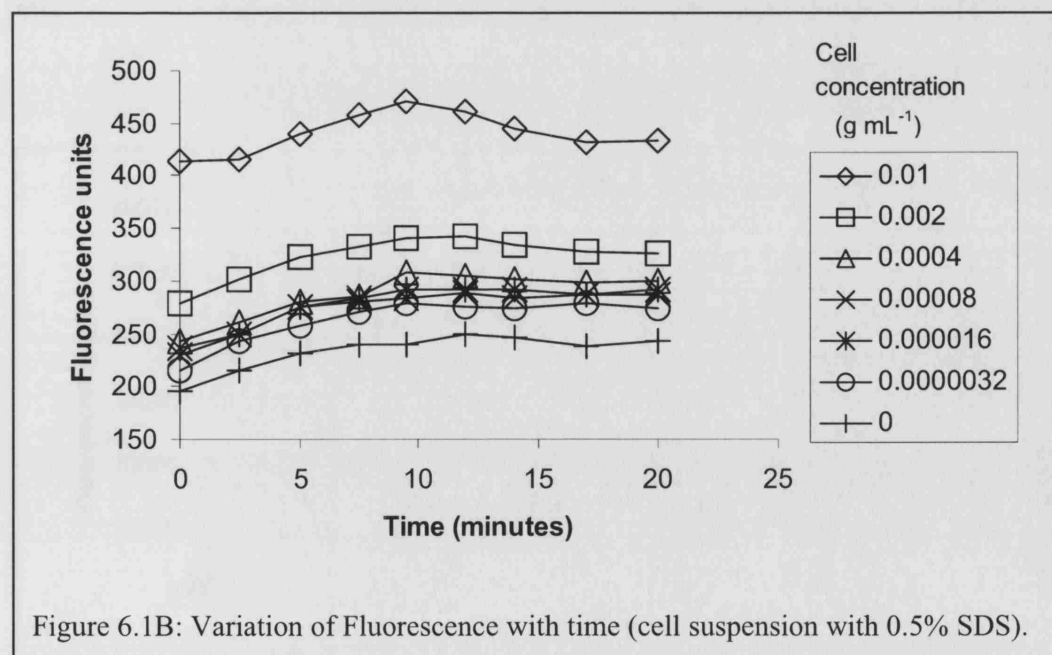
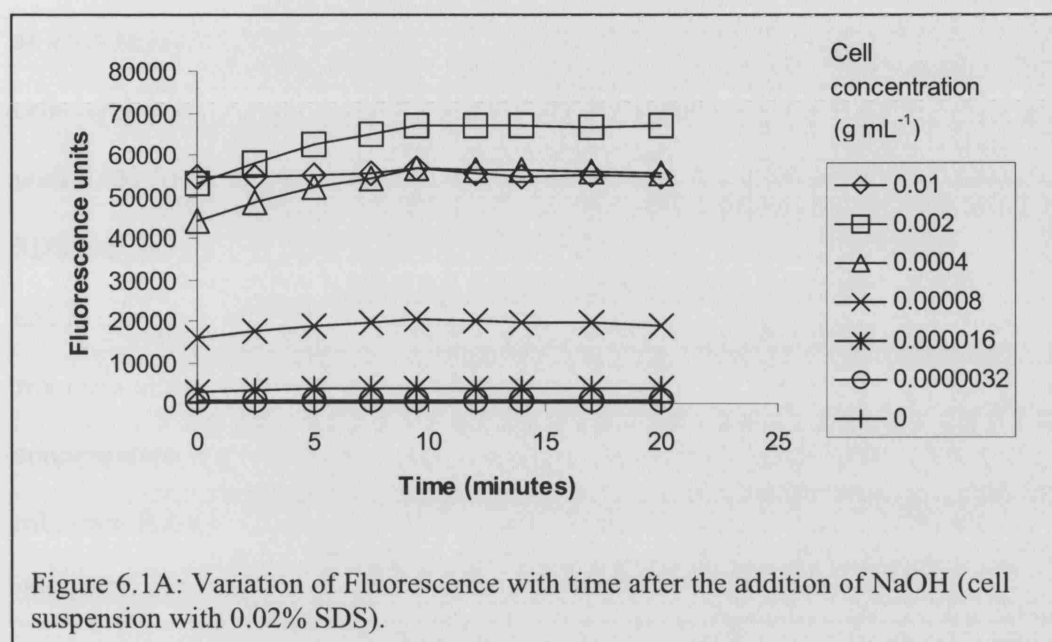
6.2 Method Development

6.2.1 Lysis Methods

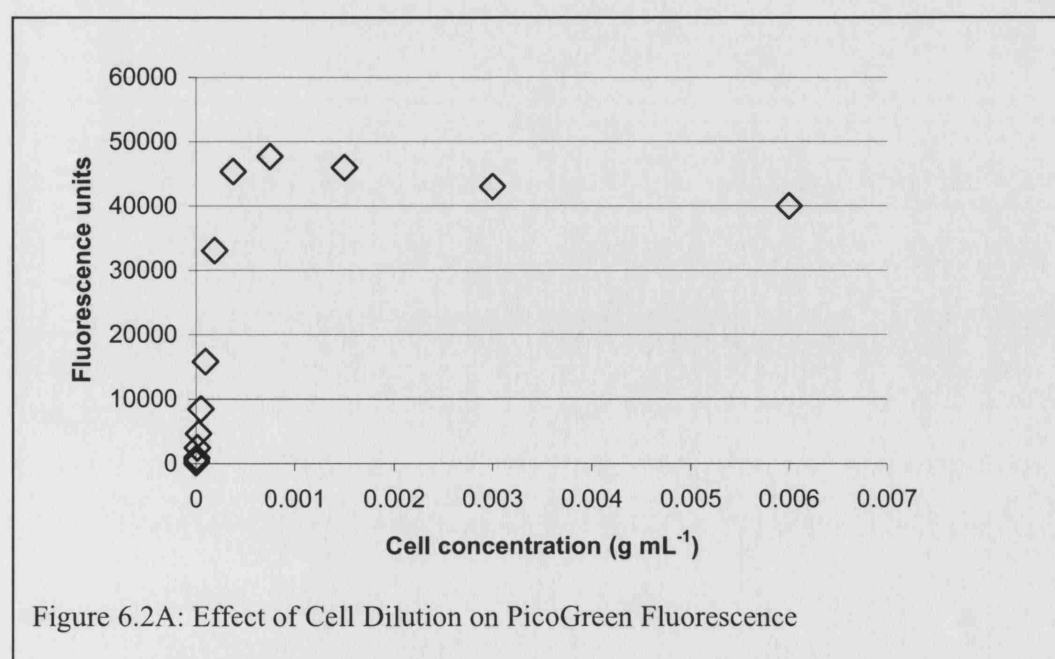
6.2.1.1 SDS and NaOH

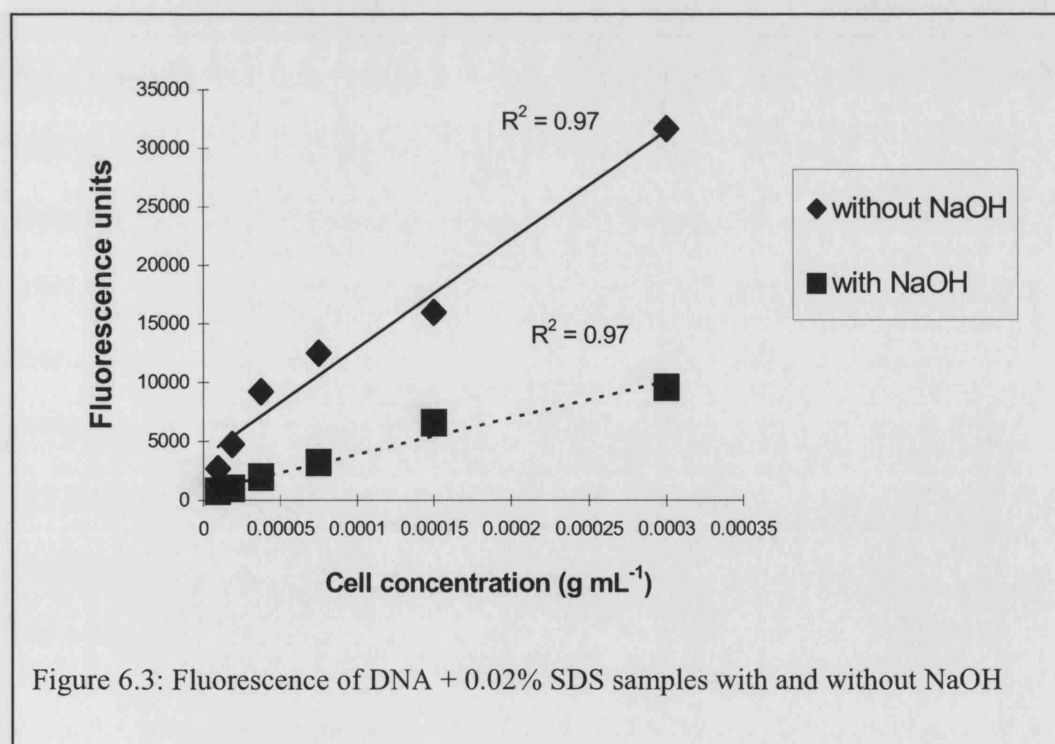
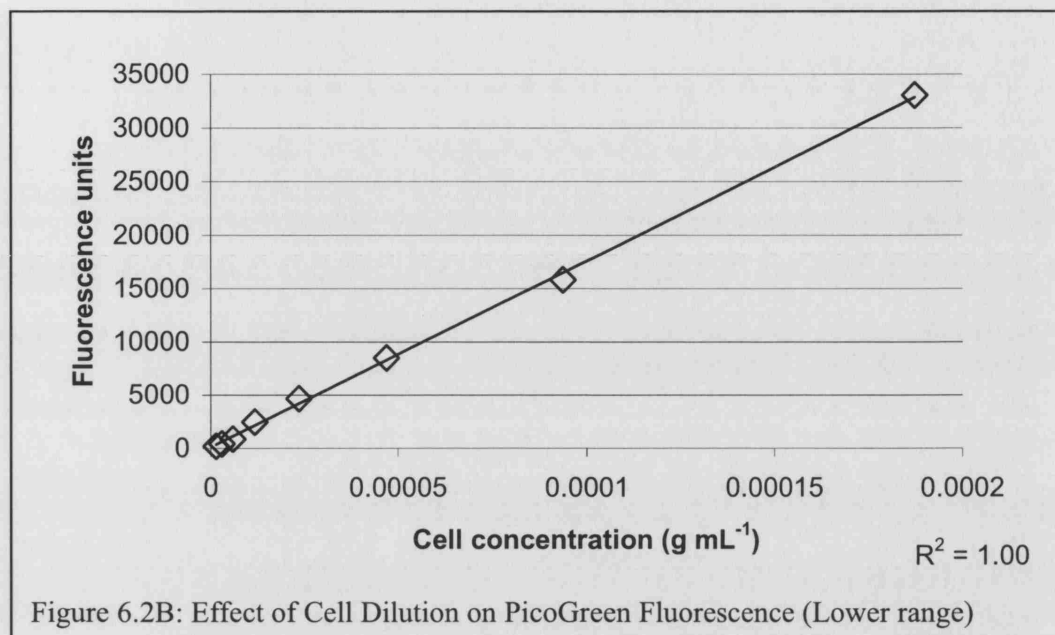
The first method considered used an analysis of crude lysates; these were made by chemically lysing cell suspensions of *E. coli* pSV β and diluting the lysate with TE buffer. This was done until the low concentrations of cellular contaminants in the mixture sufficiently diminished interference and allowed the reading of accurate fluorescence signals. Unclassified lysates allow ease of use and decreased assay time. Firstly, cells were opened using the detergent sodium dodecyl sulphate (SDS). Samples of 0.02% SDS was compared to 0.5 %, 0.1 M NaOH was added after the initial reading of fluorescence in order to denature dsDNA to the single stranded form. The decrease of fluorescence output was then monitored. The concentrations were chosen to be in line with standard alkaline lysis methods (Birnhorn and Doly, 1978). However, the fluorescence values with 0.5% SDS were very low for the dilutions tested, this indicated that the signal was considerably depressed, values seen with 0.02% SDS were higher, Figures 6.1A and B. This interference has been reported in literature (Singer et al., 1999). When 0.1 M NaOH was added, the decrease was higher for 0.5% SDS cells than for 0.02% SDS cells. Cell concentration ranged from 0 – 0.01 g mL⁻¹, but a lack of linearity in the cell concentration vs. fluorescence graphs, Figure 6.2A, indicated that concentration was too high and fluorescence signal saturation or interference was occurring. The typical cell concentration in a high yield 500 mL culture is ~4 g. A cell concentration of 0.01 g mL⁻¹ is 1g per 100mL then represents an undiluted cell suspension. The fluorescence response, however, became linear after a cell dilution of 0.0002 g mL⁻¹, Figure 6.2B. This represents a 1/50

dilution. Thus, the range of SDS concentrations was expanded and the cell concentration was decreased.



The fluorescence of cells treated with 0.02% SDS was found to have a good linear relationship over varying cell concentrations, as shown in Figure 6.3. The range of cell concentrations best for the PicoGreen was found to be 0.0003 g mL^{-1} and lower as good linearity of fluorescence response was shown in this range. To verify that the cells were being opened, a positive control was developed where cells were treated with 0.5% SDS, as used in common protocols and then diluted to the range where SDS has no interference with the PicoGreen signal, as defined in Singer et al, 1997 and in manufacturer's data. Calculation of the amount of DNA that could be released from this quantity of cells however highlighted a discrepancy, for 0.0003 g mL^{-1} cell concentration the quantity of DNA released would be on the order of picograms per mL, even factoring the RNA, which would also be released and bound by PicoGreen, this could still be a very low theoretical value. Fluorescence values, when compared to standard curves, suggest far higher values, on the order of micrograms of DNA per mL.





6.2.1.2 Lysozyme and Triton

With doubts about the correct analysis of SDS process streams with PicoGreen, an alternative method of cell disruption was investigated using $50 \mu\text{g mL}^{-1}$ lysozyme and 0.1% Triton of varying concentrations and comparing this method to SDS of varying concentrations and zwittergent. However, the use of zwittergent was found to be unsuitable in the process stream analysis. High fluorescence values were seen for the 5 mg mL^{-1} Hen white lysozyme (Ausubel et al., 2002) and 0.1% Triton, which rose with time, indicating that cells were being disrupted. After addition of NaOH, significant decreases were seen in all samples, except the negative control of unbroken cells. The optimal time for lysozyme and Triton incubation was determined from further experiments to be around five to ten minutes. The decrease of fluorescent enhancement of PicoGreen-DNA complex seen with the addition of NaOH was then monitored for dilutions of lysed cells from a large-scale (450 L) fermentation, artificially degraded cell paste, which was exposed to 37°C for 24 hours after harvest, and control cell paste from a small-scale shake flask fermentation. Lysis was carried out using lysozyme and Triton. Fluorescence values for the 450 L cell paste were greater than for the artificially degraded, which in turn were greater than the shake flask. This indicated a difference in the DNA extracted from these pastes, and showed the ability of the assay to differentiate between the extreme conditions, as shown in Figure 6.4.

To ensure that the values obtained from lysis of cell suspensions with lysozyme and Triton were giving a true reflection of the nucleic acid within the cells, values were compared to a positive control using standard 0.5% SDS lysis conditions

and diluting the lysate afterwards. A negative control of unlysed cells and a lysozyme and Triton positive control of lysed cells diluted with TE buffer were also used. The fluorescence signals of these controls were compared to the fluorescence obtained from the assay using a series of dilute cell concentrations lysed by lysozyme and Triton. Figure 6.5 shows the fluorescence of these lysed suspensions at varying dilutions. Disappointingly, the values for lysozyme and Triton lysed cells were only just above the negative control of unbroken cells indicating that at this cell dilution the method could not be relied upon to give discernible results. The SDS control, however, gave fluorescence that was far higher than the negative control and it was decided to use SDS in the lysis procedure for future experiments.

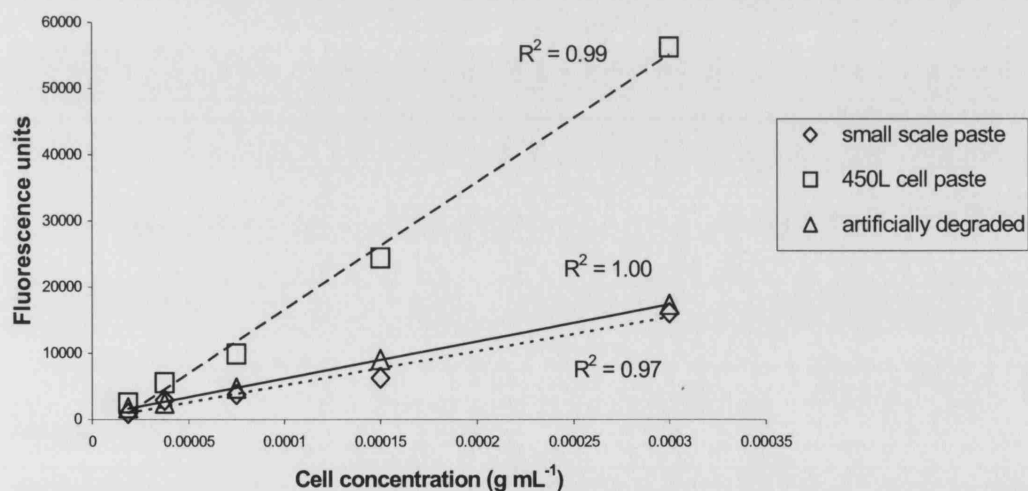


Figure 6.4: Comparison of cell pastes types using lysozyme and Triton lysis.

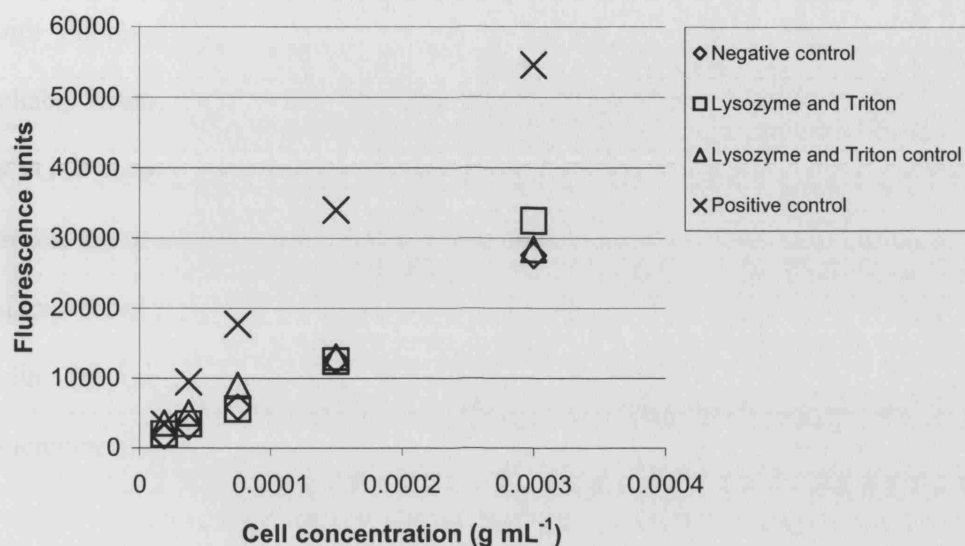


Figure 6.5: Comparison of fluorescence response of lysozyme and Triton cell lysate to positive (all broken cells) and negative (unbroken cells) controls, all $R^2 > 0.98$

6.3 Analysis of Clarified Lysates

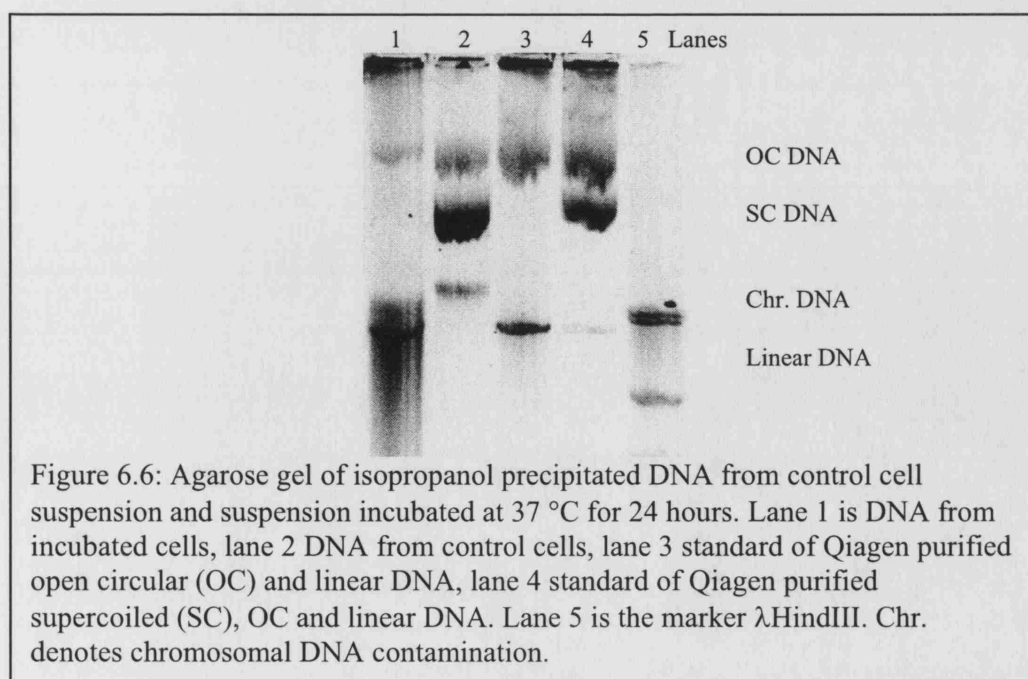
6.3.1 Introduction

The clarification of cell lysate, whether by centrifugation or by filtration, removes many of the substances which are known to cause interference with the PicoGreen signal during the analysis of crude cell lysate. The neutralisation of lysed cells leads to the precipitation of the genomic DNA, cell wall and other cell constituents. Thus, the level of these contaminants will be much lowered in the clarified lysate. After the removal of these precipitates by clarification, we are still left with a very complex mixture high in salts that may still contain traces of proteins and other contaminants. As shown in Chapter 3, the protein concentration in unclarified lysate is 0.22% which when diluted is much lower than levels that interfere with

fluorescence signal. The clarified lysate typically comprises 0.33% SDS and 333 mM acetate ions based on the methods described in Chapter 2. At dilutions greater than 1 in 100, both SDS and acetate ions are at levels below those reported as interfering with fluorescence signal, see Table 1.5. As already established, PicoGreen can reliably determine DNA concentration within clarified lysates. In this section *E. coli* pQR150 from large-scale fermentation was used to determine if the RF assay or a similar assay could be used to determine the quality of DNA. The cells in this series of experiments were lysed with SDS by standard alkaline lysis procedure and treated with RNase A to remove contaminating RNA. The lysate, clarified by microcentrifugation, was assayed at 1/200, 1/400 and 1/800 dilutions.

6.3.2 Assessment of the suitability of the RF assay

Equal quantities of the cell paste were resuspended to $OD_{600} = 50$ in TE/RNase. One suspension was degraded by incubation at 37 °C for 24 hours which significantly reduces the mass of DNA as well as the average fragment size through



enzymatic degradation, Figure 6.6, the other suspension was a control. When clarified lysate made from both suspensions was assayed, at varying dilutions, it was found that there was very good linearity of response both before and after the addition of NaOH. The resulting Relative Fluorescence (RF) readings were stable with coefficients of variation (CV) < 11%, Figures 6.7A and B, which seem to suggest that clarified lysate is suitable for further development with the RF assay.

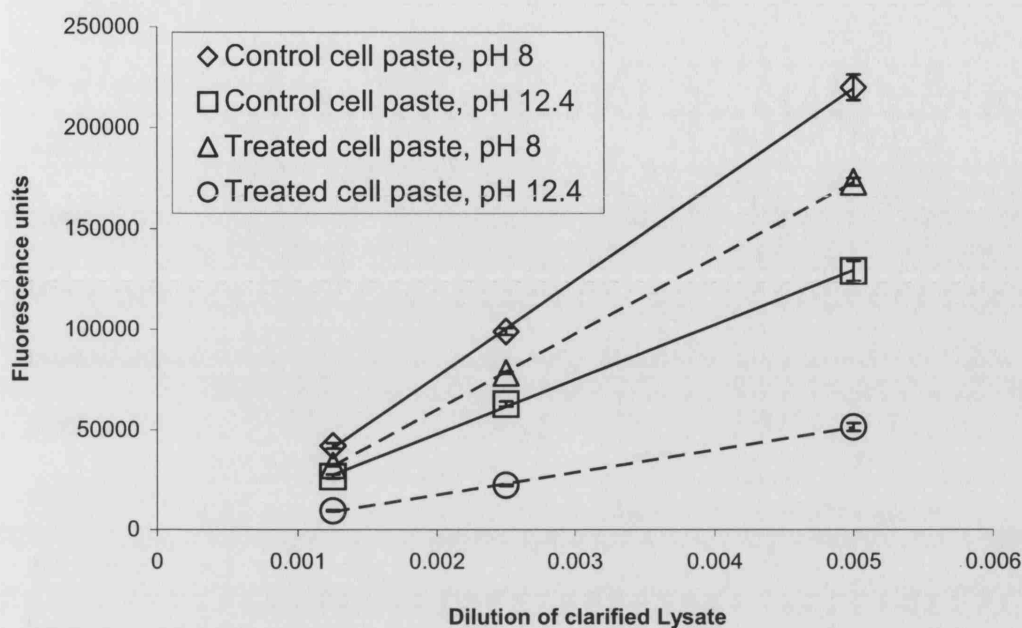


Figure 6.7A: Fluorescence response of PicoGreen and clarified lysate of cells from control and treated cell paste (degraded by incubation at 37 °C), before and after the addition of NaOH. Error bars represent the standard deviation of 18 samples.

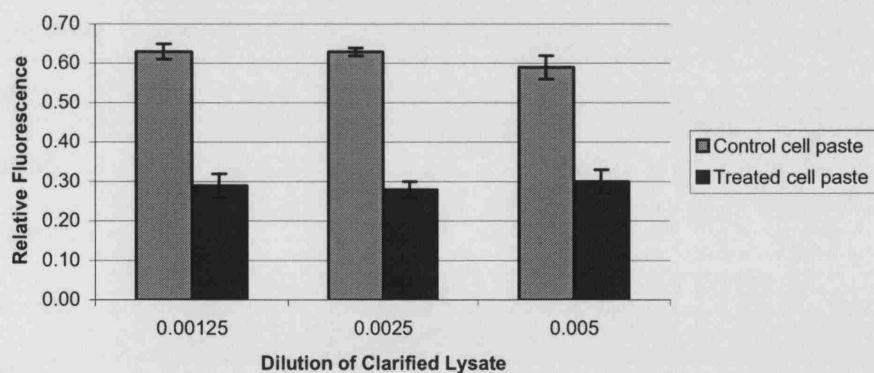


Figure 6.7B: Relative Fluorescence of clarified lysate of cells from control and treated cell paste (degraded by incubation at 37 °C), error bars represent standard deviation, number of samples = 18.

6.4 Discussion and Conclusion

The alkaline lysis step is considered one of the most challenging operations within the plasmid production process. Maximum chromosome denaturation must be attained whilst keeping plasmid DNA denaturation reversible. The rate of denaturation of the chromosomal DNA will depend on the integrity of DNA backbone. Highly nicked DNA will denature more quickly and produce a less viscous lysate before neutralisation. However, if fragments are too small they may stay in solution and not form part of the flocculant after neutralisation. As shown in Chapter 4, conditions during fermentation, centrifugation and holding can influence chromosomal integrity and hence impact downstream operations. The use of an on-line viscometer during the lysis step has been recently reported by Prather et al., 2003. Such knowledge can greatly improve the quality of the extracted plasmid product. The optimum conditions for the reversible denaturation of plasmid DNA will depend on size and probably sequence but may also depend on fermentation conditions (O’Kennedy, Glaxo Smith Kline, personal communication).

With hindsight, the preliminary lysis work should have been carried out for a range of SDS concentrations and lysozyme and Triton concentrations. From these, the lysed suspensions should have been centrifuged in order to separate cell debris and unlysed cells from supernatant containing DNA from lysed cells. The DNA should then have been precipitated and run on an agarose gel. This would have indicated at which concentrations maximum cell lysis occurred. There is also the danger that the use of the PicoGreen fluorescent dye may have been unsuitable primarily due to its affinity with RNA, although this is reported to be low by PicoGreen manufacturers (Molecular Probes, Leiden, Netherlands). It is an area where there is much scope for further development as there is a great need to develop process analytic techniques

that can adequately characterise cells throughout the lysis stage. Thus, results that were, in fact, inconclusive on the applicability of the standard PicoGreen assay for this purpose, were included.

However, despite these reservations, experimental application of the Relative Fluorescence assay to clarified lysates worked well. The variability shown was within the FDA accepted range of $CV < 15\%$ as described in the Project Background. In order to advance the investigation, further validation is warranted. Spiking tests should be carried out to determine the maximum levels of contaminants, sensitivity tests should also be done with a range of cell dilutions which would give a range of released nucleic acids to determine the minimum and maximum cell concentrations, and hence nucleic acid concentrations for the assay. There should also be correlation of the RF results to percentage supercoiled gained from an independent means. This independent method must be able to analyse crude samples. HPLC columns can be loaded with clarified cell lysate and used to accurately estimate total plasmid yield. For plasmids under 10 kb, the open-circular and supercoiled DNA forms can be separated under special operating conditions. However, for plasmids over 20 kb, isoform separation could not be achieved (Aloke Dey Choudhury, University College London, personal communication). An estimation of isoform from loading isopropanol precipitated samples onto agarose gel electrophoresis could be a suitable method.

7 AUTOMATION OF THE RF ASSAY IN THE CONTEXT OF PROCESS ANALYTIC TECHNIQUES

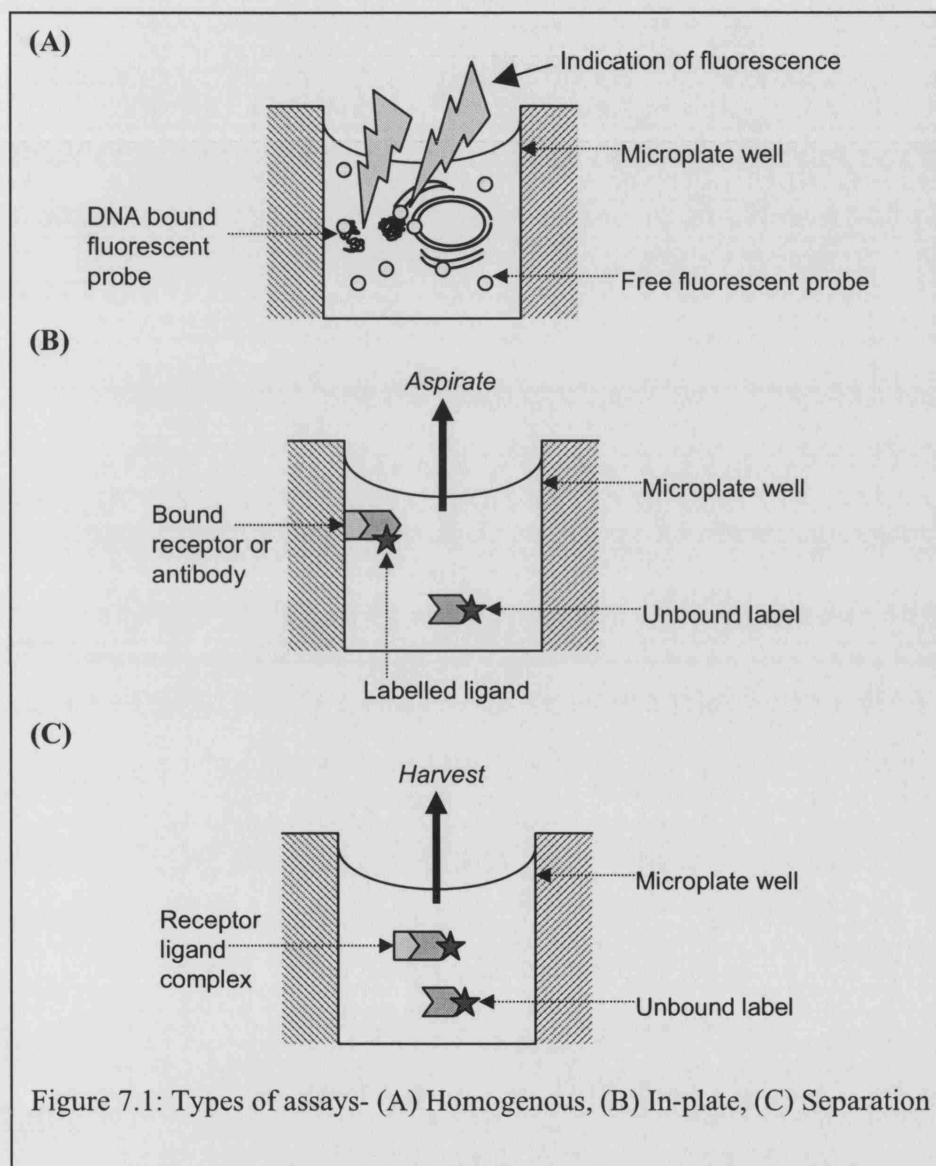
7.1 Introduction

7.1.1 Assay Automation in Industry

Advances in the automation of high-throughput screening have been made in the areas of instrumentation, robotics and assay technology. Liquid handling systems with the flexibility to utilise a variety of containers, were among the first instruments to be integrated into robotics systems (Kolb, 1994). Traditionally, analytical instruments have not been linked to robotics because of the complexity of preparing samples for analysis. Biological assays are now being developed to be less complex, require fewer separation steps and thus more amenable to automation and the high throughput of samples.

Assays can be divided into several broad categories distinguished by the number and complexity of steps involved. They may be defined as homogenous, in-plate and separation assays. Homogenous assays are simple assays that do not require extensive preparation or separation procedures. All preparation, incubation and analyses occur in the same well, as shown in Figure 7.1A, which shows a fluorescent dye such as PicoGreen, which is used in the RF assay. In-plate assays like homogenous assays may be radioactive, luminescent, colorimetric or fluorescent in nature. However, in-plate assays usually involve the use of immobilised receptors or antibodies which in turn capture label-bound antigens. Unbound ligands and labels are removed through aspiration as their presence would interfere with the measurement of bound activity, shown in Figure 7.1B. Separation assays, Figure 7.1C, require a step to separate bound from free label. The mixture may be harvested using a filter which retains the receptor bound ligand but allows the unbound ligand to pass through. In

order for assays to be more amenable to automation they should be either in-plate or homogenous in nature, thus mitigating the previously rate limiting step of assaying and allowing higher throughput rates and greater efficiency.



7.1.2 Automation in the context of PAT

Process Analytic Techniques (PAT) have recently been publicised by the United States Food and Drug Administration (US FDA) as the new system for the validation of pharmaceutical processes. PAT are systems for the analysis and control

of manufacturing processes based on measurements of critical quality parameters and performance attributes to ensure acceptable end-quality of pharmaceutical products. Within PAT, robotic liquid handling systems along with integrated analysers, such as microplate readers, may represent an efficient and economic method of conducting the measurement of critical parameters. The liquid handling device allows micro-scale studies to be conducted at a high number of process conditions, resulting in maximum data collection using minimum amounts of sample and reagent. The flexibility and rapidity of the robotic system also fits easily into the plasmid purification process where it may become an integral part of the validation process.

7.1.3 Overview of RF work and automation methodology

So far in the body of work, a manual definition of the RF assay has been completed and the validation of the assay with reference to purified solutions of 6.9kb, 20kb plasmids and bacterial artificial chromosomes. The precision, ruggedness, robustness and the accuracy of the manual assay have been evaluated and work has also been extended to impure solutions and whole cells. Automation of the RF assay was pursued for the following reasons. It was considered that automation of the assay affords increased capability, thus more assays can be carried out in less time. There will also be increased reliability with less error and scope for contamination, thus also resulting in greater reproducibility of the assay. Manually a 96 well microplate may be assayed in 10-12 minutes; this may be considered rapid but automation also affords greater flexibility for expansion and as the number of well and microplates increase the time savings offered by automation will be more apparent. The RF assay is already rapid and cost effective as it uses very little sample and reagent quantities; however, extension of the assay to the robotic liquid handling system should

substantially increase the precision, sample turnover and robustness of the RF assay. Thus, the adaptation of the present manual assay to the robotic liquid handling system is a crucial step in assessing its suitability for its proposed purpose in industry.

Within this chapter, the RF assay conditions (pH and time) will be re-established for an automated liquid handling system with reference to the 6.9 and 20 kb plasmids. The equivalence of the manual and automated methods will then be evaluated and the adapted assay will be optimised and validated.

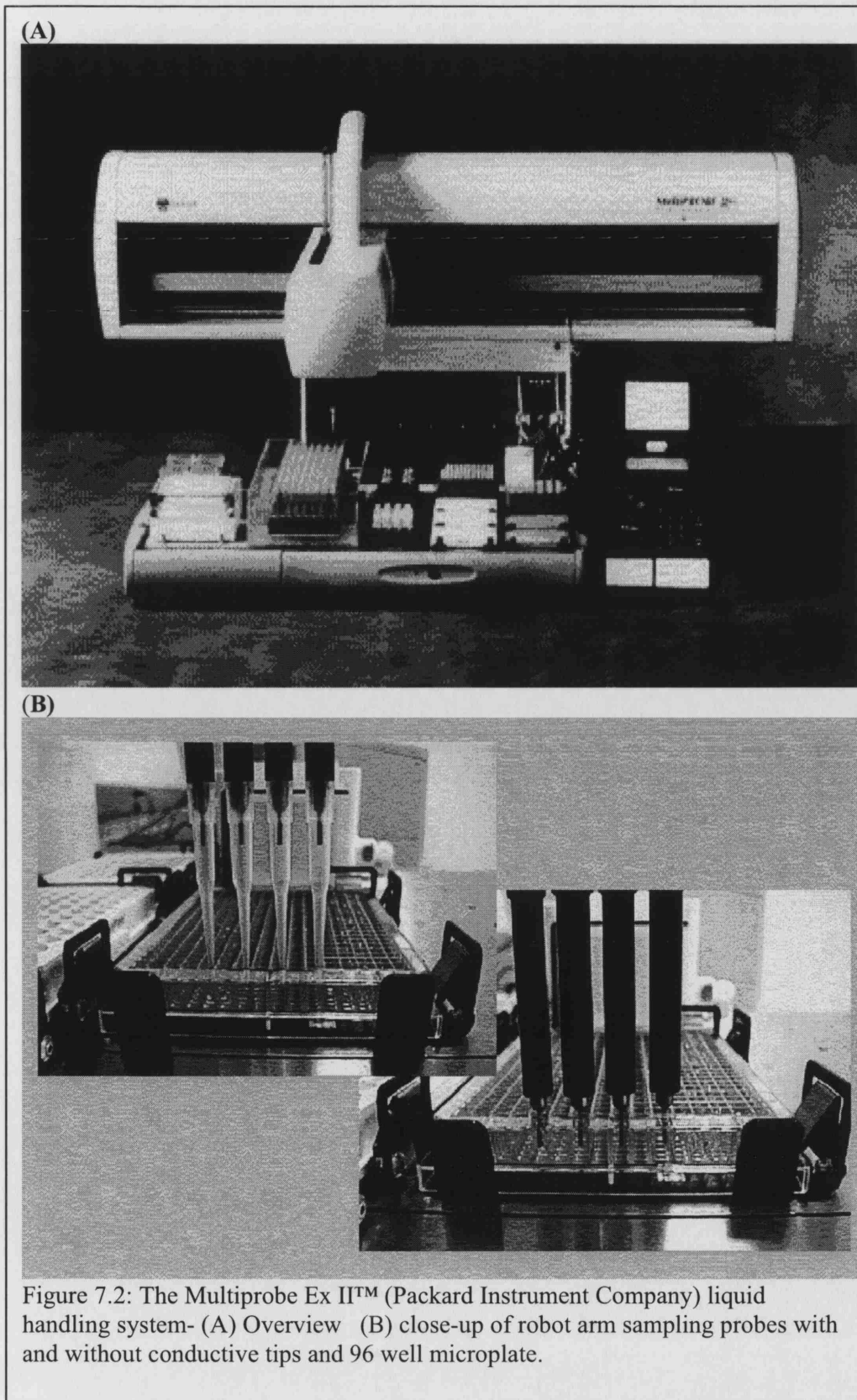
7.1.4 The Multiprobe Ex II™ Liquid Handling System

The liquid handling system is shown overleaf in Figure 7.2.

The trend towards assay miniaturisation has been assisted by the advent of automated liquid handling solutions such as the Multiprobe series (Packard Instrument Company, 800 Research Parkway Meriden, CT, USA). Automated liquid handling applications require a wide dynamic range of volume dispensing capabilities from robotic pipettors. Automating DNA purification and high throughput screening applications, however, require the addition of larger volumes across a 96 well plate or into a deep well plate.

Automation of nucleic acid purification and analysis increases sample processing throughput and decreases the contamination and variability frequently associated with manual operation. Nucleic acid purification is an important rate limiting step in large-scale genomic sequencing and micro-array production efforts. All liquid handling steps are controlled by the easy to use WinPREP™ software.

There has already been reported use of the Multiprobe Ex II™ in the automated purification of plasmid DNA as well as high throughput fluorescent quantification using PicoGreen (Poineal et al., 2002; Tack et al., 2002).



To meet these demands, a number of new technologies have been developed to allow the Multiprobe Ex II™ to be used to pipette a wide variety of volumes in a reproducible and sensitive manner. These technologies utilised by the Multiprobe Ex II™ are described below.

The Multiprobe Ex II™ offers tip technology for volumes ranging from millilitres to nanolitres, positioning reproducibility for high density labware such as 384 well microplates, thus enabling very high throughput of samples. The Multiprobe Ex II™ also offers superior liquid sensing performance, as described by the manufacturer's literature.

With the integrated Windows NT® applications software, programming and assay set-up is relatively uncomplicated due to drag and drop commands, availability of standard and custom procedure templates and the compatibility with custom macro scripts.

Through the unique modular deck design, assay set up is simplified and positioning reproducibility is enhanced. Contamination risk may also be mitigated through choice in the uptake and dispensation type. There is also the ability to integrate ancillary equipment within the pipetting area, thus the automated capability of the assay may be extended.

Using patented technology, the Multiprobe Ex II™ also offers superior liquid level sensing performance down to 50 µl per microwell; it also enables both ionic and non-ionic liquids such as DMSO or methanol to be detected. With accurate sensing the over-immersion of sampling tips is minimised and improvement in pipetting performance is achieved. This is more effective than other sensing techniques such as Tip Touching Techniques.

The Multiprobe Ex II™ is also able to dispense accurately both without conductive tips and with either washable or disposable conductive tips. Varispan™ tip separation allows computer control of variable sample probe spacing for automation of different methodologies regardless of the combination of containers used. Sampling probes can vary in spacing from 9 mm to 20 mm allowing the use of test tubes, microplates, deep well plates and vials in various combinations in the pipetting area.

Contamination between samples is mitigated through the use of washable Teflon coated stainless steel sampling tips. Modular deck design provides enhanced liquid handling performance through deck calibration and standardisation, high quality labware support tiles and capability of labware rotation to ensure flexibility of layout.

The WinPREP™ operating system has the unique capability to calibrate pipetting performance for volume ranges, fixed tip, disposable tip, waste mode and blow-out mode combinations. The software uses Performance Files which utilise specific accuracy calibration and settings for aspirate and dispensate speeds, waste volumes, transport and system air gaps, blow-out volumes, blow-out delays and waste volumes. The WinPREP™ application also contains a predefined labware library thus enabling users to select a piece of labware, then drag and drop it to any location on the deck. Procedure templates are available for standard pipetting operations such as single and multiple liquid transfers, reagent additions, pre-dilutions, serial dilutions and re-formatting procedures. Pipetting patterns can also be easily defined by selectively clicking on specific wells or groups of well in the order of processing, the default may also be utilised. File based selective lists allow the modification of prior programs and reformatting of secondary assay plates. Custom procedures can easily

be created through the linking of basic pipetting commands such as aspirate, dispense, flush, wash, get tip and drop tip and move to. Each of these commands can also be modified to reflect concerns such as contamination, for example, to minimise this risk one could opt for the use of tips with single aspiration per dispensation. For dispensation of low risk liquids such as water, a single aspiration for multiple dispensation mode may be used without the use of conductive tips.

Performance testing of the Multiprobe Ex II™ found that at 5 µL with 20 µL disposable tips, dispensation precision was 1.38% CV (<3% specification) and inaccuracy of 0.97% (<1% specification), (Ahrweiler et al., 2002). Other assay applications for the Multiprobe Ex II™ include modified HPLC and urine constituent determination (Musson et al., 2002).

7.2 Adaptation of the manual RF assay

7.2.1 Programming of the liquid handling system

For the purpose of the adaptation of assay to the robot it was necessary to devise an algorithm or a precise set of steps that the robot must carry out. This is used in the programming of the robot's movement with the WinPREP™ programme. This algorithm was developed to perform the assay after the standard WinPREP™ initialisation and flushing which is carried out at the start of every experiment. The robot is also regularly maintained in accordance with the manufacturer's instructions and calibrated on a weekly basis. The proposed algorithm is outlined below:

1. Take up tips
2. Take up TE buffer - 60 µl
3. Deposit in defined wells in microplate for use as background readings
4. Remove tips

Repeat steps 1- 4 to complete all background wells

5. Take up tips
6. Suck up 60 µl of DNA solution
7. Place DNA in defined cells in microplate
8. Dispose of tips

Repeat 5- 8 until all DNA samples are set out

9. Take up tips
10. Suck up 60 µl PG working solution
11. Place PG in defined wells in microplate
12. Dispose of tips

Repeat 9- 12 until PG is added to all DNA

13. Transfer microplate to reader
14. Shake plate
15. 5 min delay
16. Read microplate
17. Take microplate out of reader
18. Take up tips

19. Pick up 120 µl NaOH
20. Deposit in defined cells in microplate
21. Dispose of tips

Repeat 18- 21 until all NaOH cells are completed

22. Take up tips
23. Pick up 120 µl TE buffer
24. Deposit in defined cell

25. Remove tips

Repeat until TE is added to all defined wells, if necessary

26. Transfer microplate to reader

27. Shake plate

28. Start time delay countdown

29. Read microplate

30. Remove microplate from reader

31. Transfer to start position

Within this sequence of steps, some aspects could be varied for the purpose of speed and reliability. The first consideration was whether the assay should be carried out in two parts, where DNA is dispensed with PG read and then NaOH added. This may be shortened to adding NaOH to some wells and TE to others, but besides the halving of samples that can be tested; there is the significant issue of method equivalence and variation. It was decided not to introduce this added element of variation to the assay and to maintain the current two part assay structure. It was also estimated that shortening the assay time by truncating the dispensation will only save 1- 2 minutes for a 96 well microplate and this was not considered beneficial enough to warrant the change in method. The original manual RF assay was dispensed in 50 μ l aliquots. This volume was increased in the automated assay to 60 μ l aliquots as this effected a substantial increase in the mixing, dispensation accuracy and reliability of the Multiprobe Ex II™ (Nealon et al., 2004). In his assessment of the suitability of a liquid handling robot integrated with an analytical device for gathering kinetic data on a process step, Nealon showed, by measuring standard ultrapure water samples, that dispensation post calibration successfully conforms to the manufacturer's

specifications. The use of a liquid handling robot thus allows micro-scale studies to be completed, allowing large amounts of data to be collected with minimum sample and reagent use.

7.2.2 Assay optimisation methodology

In the optimisation of the time and performance of the RF assay, the first step was to determine of the sequence of the assay. The resting time of DNA samples with alkali was considered the most important assay parameter and was thus addressed first. The possibility of decreasing alkali strength was also considered, in order to make the assay more reliable and accurate. Once the adapted assay parameters were attained the validation was commenced with regards to accuracy and variability. The plan of experiments conducted is outlined below:

- Determination of experimental pH and assay volumes.
- Determination of correct step sequence and comparison with historic manual data
- Monitoring of fluorescence signal with time
- RF response to varying pH
- Demonstration of manual and automated method equivalence
- Assay sensitivity experiments using mixtures of chemically degraded and control DNA.
- Determination of adapted assay precision and variability

7.3 Methods

7.3.1 Plasmid Purification Reference

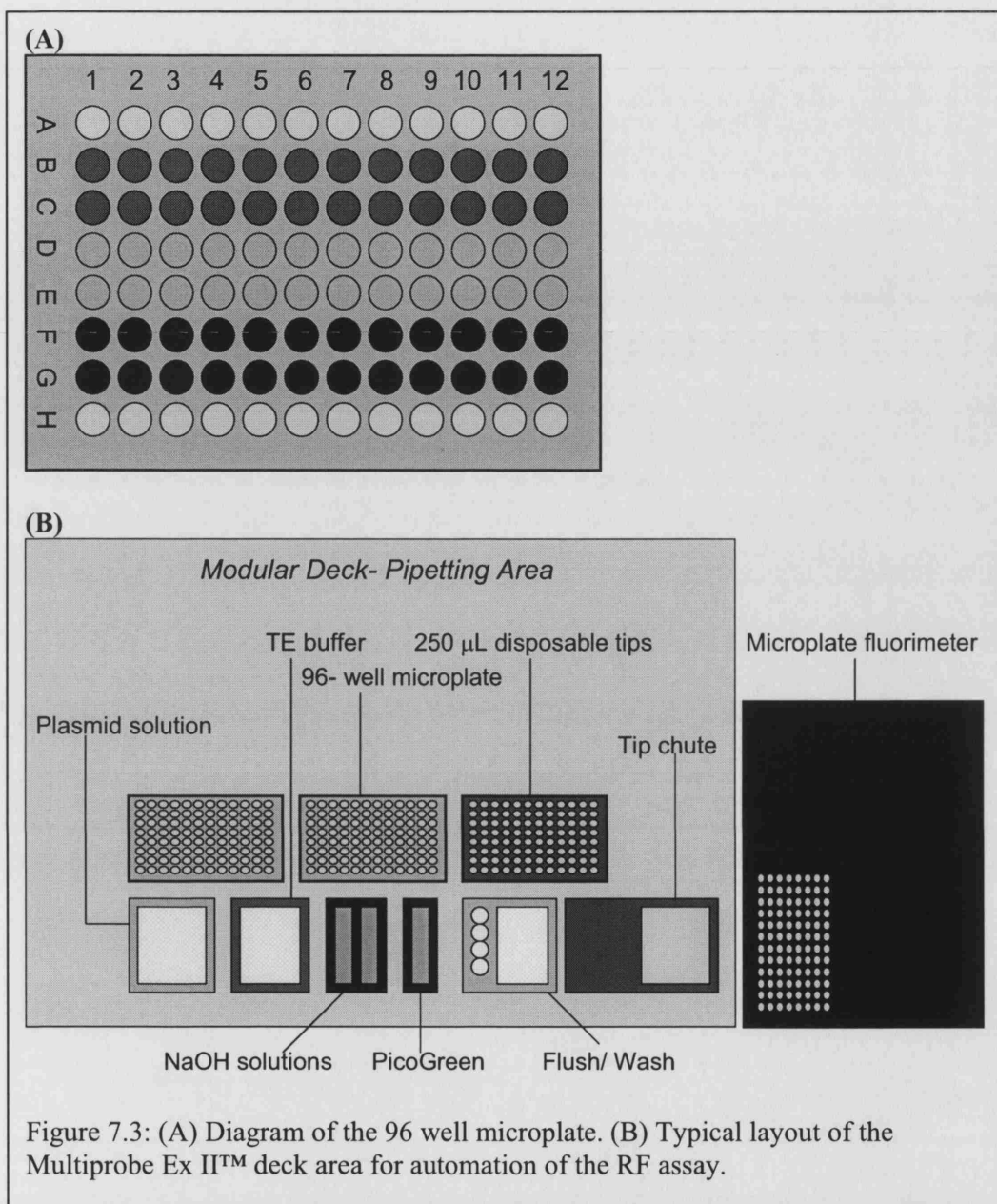
The methods and materials used to produce pure samples of plasmid DNA and BAC are defined in Chapter 2. The standard RF assay is also defined within Chapter 2.

7.3.2 RF Assay Automation

The four tip Packard Multiprobe Ex II™ robotic workstation (Packard Instrument Company, Meriden, CT, USA), WinPREP™ software and integrated plate reader were used. DNA (60 µl) and PicoGreen (60 µl) were added to each well in single dispensation per aspiration mode, the 96 well plate was shaken for 5 sec in the FluoroCount, incubated for 5 min and read. The plate was transferred to the workstation where NaOH (120 µl, to pH 12.4) was added via multiple dispensation per aspiration mode, shaken for 5 sec, incubated for 5 min and read.

7.3.3 Final protocols for the assay operation with the Multiprobe Ex II™

The final detailed protocols used for the automated operation of the RF assay are outlined below, the typical layout of the deck area and 96 well microplate is shown in Figure 7.3:



Protocol 1: Before Operation of Multiprobe Ex II™

- 1 If testing a new programme, fill reservoirs with water, do not use designated PicoGreen reservoir.
- 2 If executing a trial, prepare DNA, NaOH and PicoGreen (PG) working solution (1/ 200 dilution of PG stock in TE) samples in disposable containers. Calculate necessary volumes beforehand and add around an extra 10%, allowing requisite time for defrosting of DNA and PG stock. Store covered samples at 4°C until needed, but not for more than 6 hours.
- 3 Measure pH of NaOH stock and carry out test dilution to ensure that resulting pH in microplate well will be the required pH.
- 4 Wash and dry all sample reservoirs with towels, rinse with TE and fill with samples. Use only the designated PG reservoir and lid for PG.
- 5 Lay out the lab-ware on the Multiprobe deck. Put the reservoirs in their holders and put lids on to minimise evaporation while setting up the Multiprobe Ex II™ programme.

Protocol 2: Programming and Running of Multiprobe Ex II™

- 1 Set up the fluorimeter, paying attention to the lines marked on the test area, locate and connect the power cable and source, cable to computer and the light source cable to fluorimeter and light source power cable. If the fluorimeter is already connected, double check the named cables.
- 2 Turn on the computer, Multiprobe deck, fluorimeter and light source.
- 3 Enter the password into the computer and wait for it to start Windows.
- 4 Check the height of the system fluid (water drum beside the robot), if level is below the sensor, refill with ultra pure water.

- 5 Check that the tube from the flush/wash water reservoir goes into the barrel on the floor.
- 6 Enter the Multiprobe programme through WinPREP™.
- 7 Load the Flush / wash programme.
- 8 Go to utilities → instrument → initialise to set the x and y co-ordinates of the pipettes.
- 9 Execute Flush / wash test (6ml); get rid of bubbles by tapping the flexible tubes and then tapping the bottom of the rigid tubes.
- 10 Leave the Flush/wash program, remember to ensure the water reservoir drains and does not overflow.
- 11 Load the PicoGreen program.
- 12 Check that all labware is in correct position on the grid.
- 13 Double-click on the microplate, check and position corners of plate, top left and bottom right, 1st well, 1st column and last well, last column, check and position well bottom and well top. The motor should be turned off before adjustment.
- 14 Turn on Macro Express and Plate Reader programs.
- 15 Make a note in the PicoGreen program of where the reservoirs for each reagent are located, which will help with program amendment and parameter setting, if necessary.
- 16 Carry out trial if no amendments are to be made by selecting EXECUTE. Remove lids from reservoirs and close the hood before commencing operation of the robot.
- 17 Put in extra steps if necessary, save the PicoGreen program as the trial name (1, 2, and so on) so that a copy of the program is retained. Never alter the

original programme. Use the third row of icons to add a step whether it is reagent, single liquid addition, flush / wash etc. Alternatively, highlight a step to be copied and drag it down to the new position. Click on the step and amend the stages / parameters highlighted in bold – i.e. say where tips and liquid will be taken from, where liquid is taken to and so on. Note that in the microplate the exact wells to be filled must be entered and even when steps are copied all the parameters must be checked.

- 18 Execute each step of the amended program by saving a new test and deleting all steps other than the one that you wish to test, use the reusable tips and water. Ensure that the hood is closed before commencing operation of the robot.
- 19 When each step is verified to be accurate, execute the entire programme with reusable tips and water. If the robot must be stopped, use the emergency stop button on the deck and fix the problem and then reset the deck with reagent and tips
- 20 When tests are finished close the programme or carry out experiment as directed previously.

Protocol 3: After the Experiment

- 1 Load the PG cleanup programme, check position of PG reservoir and amend if necessary. EXECUTE. This should take all waste from the microplate and put it back into the PG reservoir. Put the lid on the PG reservoir.
- 2 Empty all other liquid wastes in waste barrel.
- 3 Dispose of the PG waste in designated bottle containing activated charcoal. Make sure to drain fully, wipe with tissue and dispose of tissue in the nearest yellow bin bag.

- 4 Remove all lab-ware from the robot deck and wash reservoirs in sink and put to dry. Make sure to wash PG container and lid separately to minimise any cross-contamination.
- 5 Shut Cleanup programme and load Flush / wash programme and execute.
- 6 Check drainage of waste water and wipe the deck clean (*if any PicoGreen spills have occurred, refer to the decontamination procedure*)
- 7 Save any files created by fluorimeter, export to Excel and save to disk as an Excel spreadsheet.
- 8 Turn off fluorimeter, robot and light source, close hood and shut down computer.
- 9 Log date, name, materials used etc. in the logbook.

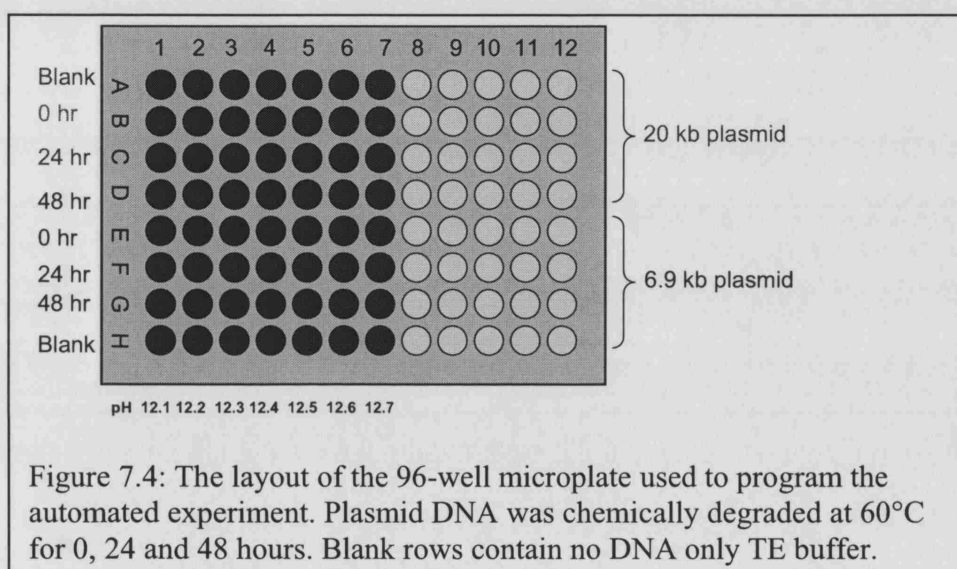
7.4 Results

7.4.1 Development of assay conditions for the automated assay

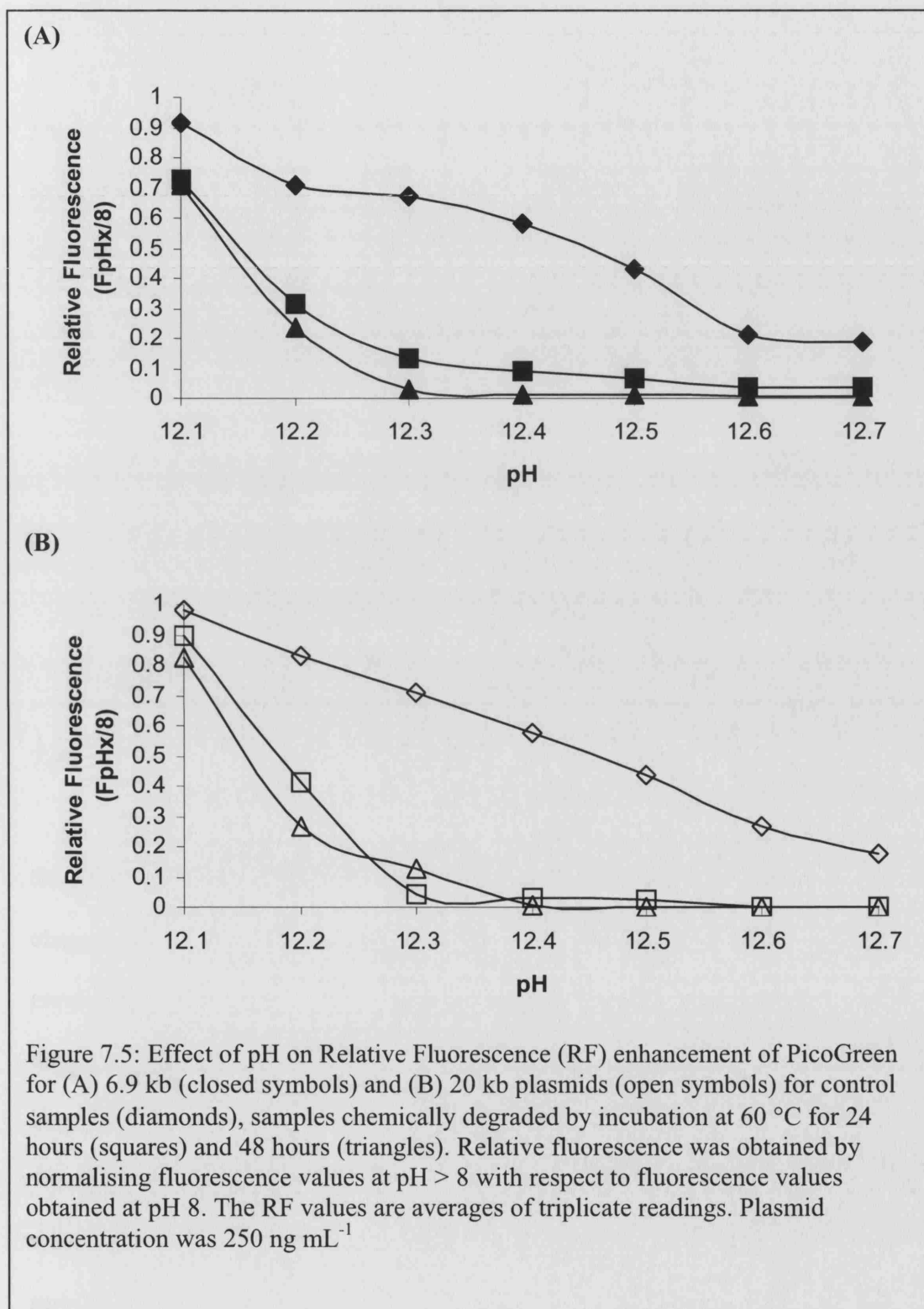
As described in the previous chapter, PicoGreen dye preferentially binds to double stranded DNA rather than the single stranded form of DNA or RNA (Singer et al., 1997). Additionally, the level of fluorescent enhancement of the PicoGreen- DNA complex is dependent on DNA conformation whereby the open circular and linear forms yield higher fluorescence signals when compared to the supercoiled form (Levy et al., 1999a). Thus the PicoGreen dye, whilst useful for assessing the quantity of DNA, may also be used to indicate the integrity of the plasmid DNA form. The assay developed, described in the prior chapter, determines the integrity of plasmid DNA through the addition of alkali which causes the progressive denaturation of DNA starting at any points of single strand breakage or nicks. From previous experiments, assay time, pH, robustness and precision were determined. For the automated version of the assay, these conditions must be re-established, optimised and equivalence of the assay demonstrated using 6.9 and 20 kb plasmid. The first of these conditions to be re-established was pH.

7.4.1.1 Analysis of plasmid response to pH using the Multiprobe Ex II™

Prior experiments have established that denaturation of double stranded DNA commences around pH 12.1 and is complete by pH 12.9. Samples of the 6.9 and 20 kb plasmids, purified and chemically degraded according to the methods established in the prior chapter, were subjected to pH conditions from 12.1 to 12.7. This was done to confirm the effective range for the automated operation and assess the robustness of the assay. The microplate layout used for the experiment is shown below (Figure 7.4):



The results found agreed with previous findings whereby fluorescent enhancement was found to decrease with increasing pH values. The rate of decrease is greater with increased degradation of plasmid DNA, as shown in Figure 7.5.



It was found that the denaturation patterns were also similar to prior experiments. Accordingly, it was decided to maintain pH 12.4 as the assay condition for the automated experiment. The greater amounts of variation seen with degraded samples are also shown in these results as they were in Chapter 5. The results also indicate the sensitivity of the assay response to changes in pH. Table 7.1 shows that changes between pH 12.3, 12.4 and 12.5 are discernible with greater than 10% CV.

		% Coefficient of Variation (CV)	
		pH Range	
		12.3 - 12.4	12.4 - 12.5
pQR150	0 hr	14.53	19.45
	24 hr	27.08	127.86
	48 hr	129.19	141.42
pSV β	0 hr	10.23	21.59
	24 hr	28.00	21.62
	48 hr	60.11	15.23

Table 7.1: Sensitivity of Fluorescence enhancement for pQR150 (20 kb plasmid) and pSV β (6.9 kb plasmid) to changes in pH.

7.4.1.2 Time analysis

At pH 8, fluorescence values were steady for over 20 minutes. However, with the addition of alkali the fluorescent signal emitted may depend on the length of time elapsed after addition. This is of great importance, not only for the determination of incubation time per microplate with the automated assay, but also for the determination of how many plates or samples may be assayed at once as the incubation time will become a constraint for the automated processing time. Figure 7.6 shows the variation of relative fluorescence from 0 to 17 minutes. These values were also similar to those gained from prior manual experiments for identical samples. Decrease in Relative Fluorescence is rapid from 0 to 3 minutes after the addition of alkali, then steadier for readings taken at subsequent time intervals. Between 3 and 11 minutes the coefficient of variation was below 10%. To further

validate this, samples were subjected to prolonged incubation at 60°C for 24 and 48 hours. For the time frame between 4 and 10 minutes the coefficients of variation were within 10% for control and degraded samples as shown in Table 7.2 below.

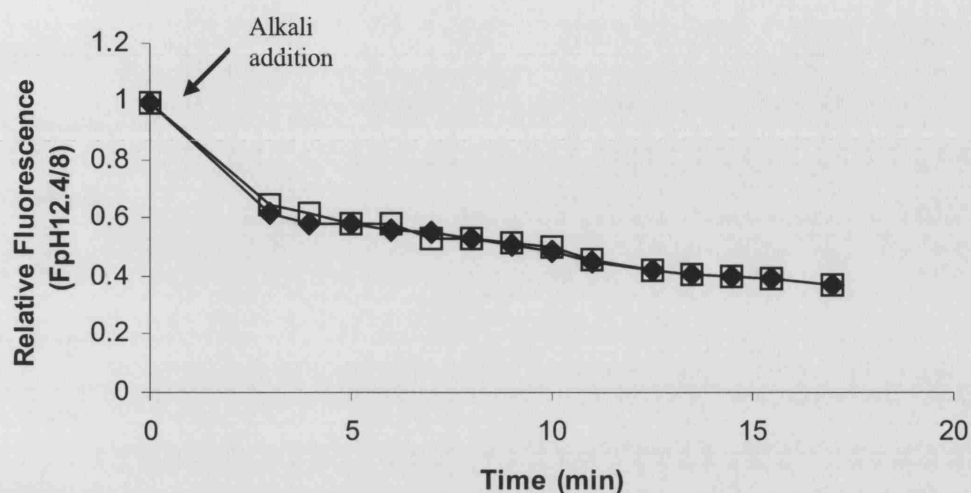


Figure 7.6: Effect of incubation time with PicoGreen at pH 12.4 on Relative Fluorescence, RF, for control 20 kb (open squares) and 6.9 kb (closed diamonds) plasmid samples. Plasmid concentration was 120 ng mL⁻¹. RF was obtained by normalising fluorescence values obtained at pH 12.4 by those obtained at pH 8.

	pSVβ (6.9 kb) plasmid		
	Control plasmid	24 h degraded	48 h degraded
RF Average	0.548	0.083	0.011
SD	0.051	0.007	0.001
%CV	9.29	9.61	9.86
Assay Time	3 - 11 min	3 - 12.5 min	4 - 10 min
RF Average	0.569	0.088	0.012
SD	0.022	0.004	0.006
%CV	3.84	4.73	4.68
Assay Time	4 - 8 min	3 - 8 min	4 - 6 min

Table 7.2: Summary of Standard deviation (SD) and Coefficient of variation (%CV) for the control and degraded 6.9 kb plasmid samples subjected to the Relative Fluorescence (RF) assay.

To ensure the validity of the assay, a CV < 10% would be desirable, based on the experimental data, the assay length should be between 4 and 10 minutes. Thus,

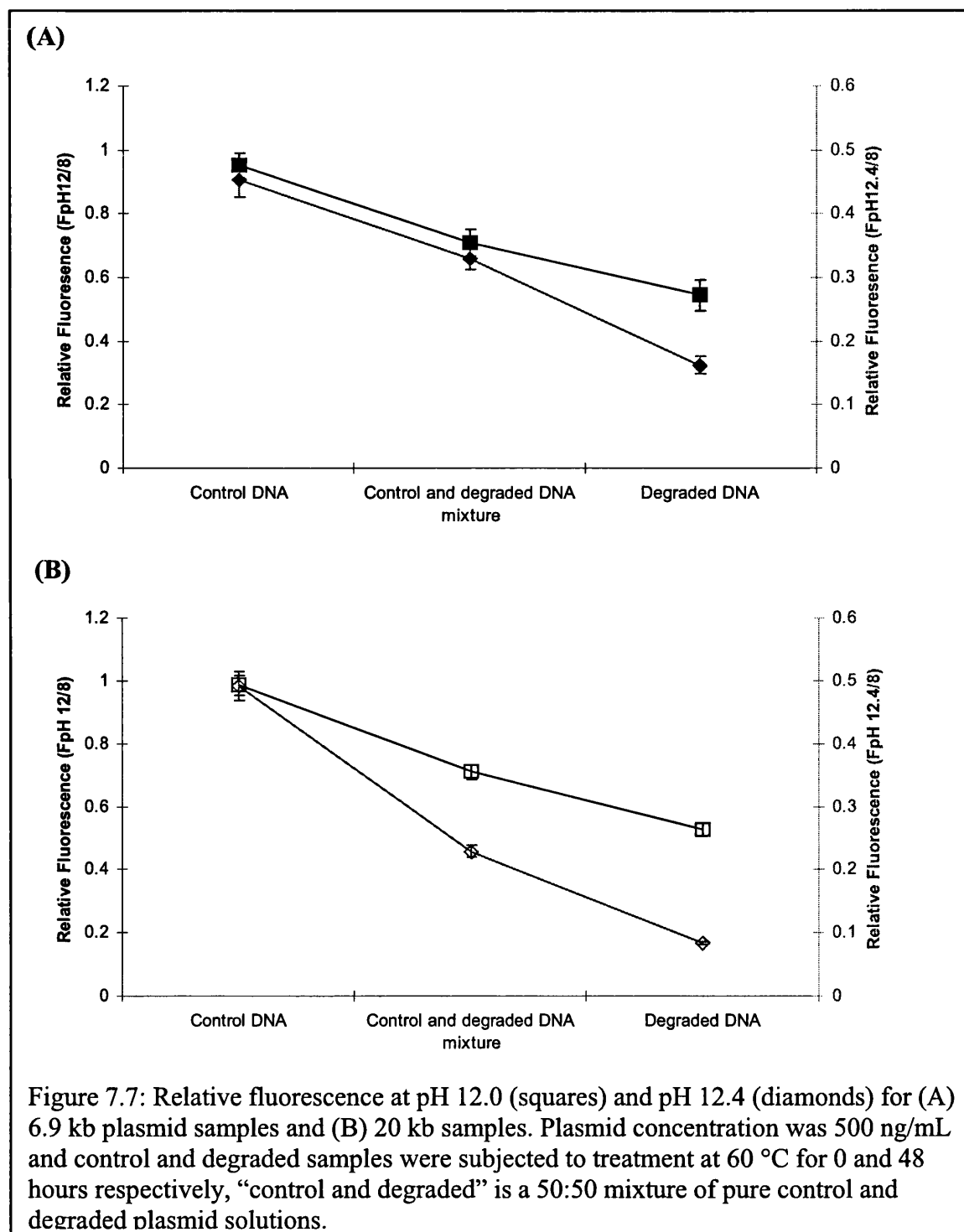
after the addition of alkali, incubation time should not be allowed to proceed further than 10 minutes.

In terms of the liquid handling speed of the 4-probed Multiprobe II EX, where the average time taken for each alkali dispensation is 5 seconds in multiple dispensations per aspiration mode, it may be seen that in 10 minutes a maximum of 5 microplates can be dispensed with alkali. Therefore, at the most, 5 microplates can be processed simultaneously before reading of the first dispensed microplate is required. Of course, the flexibility of the WinPREP™ operating program allows the pausing of the program to remove completed microplates from the pipetting area; these can then be read with minimal disruption to the automated assay, thereby enabling the simultaneous processing of in excess of 5 microplates. The speed of the actual reading of fluorescence enhancement by the fluorimeter was not found to be rate limiting to the overall operation.

7.4.2 Application of the Automated RF Assay to Degraded Samples

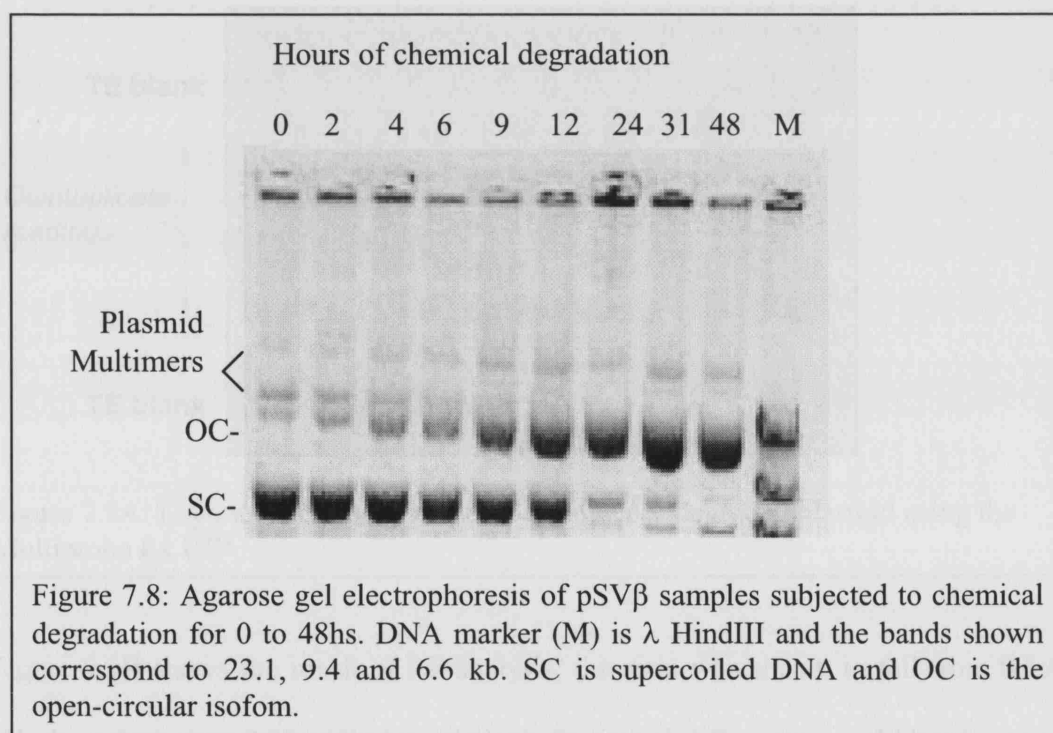
The automated assay was further extended to 6.9 and 20 kb samples which were treated with chemical degradation at 60°C between 0 and 48 hours to promote single strand breaks. As a preliminary experiment on pH suitability for control and degraded 6.9 and 20 kb plasmids, samples treated for 0 and 48 hours were subjected to the adapted automated assay at pH 12 and pH 12.4. The results are shown in Figure 7.7 A and B for plasmids of 6.9 and 20 kb respectively. These samples were identical to those used for prior manual operation of the assay, as shown in the resulting agarose gel, Figure 7.8. Densitometric analysis of the gel showed that supercoiled DNA content ranged from 88% (control) to 17% (48 hour degraded). The preliminary studies revealed that when subjected to the adapted RF assay, results for the degraded

and 50:50 mixtures of control and degraded DNA varied from 0.15 to 0.9 for 6.9 kb plasmid and from 0.1 to 0.98 for 20 kb plasmid, both at pH 12.4. These results compared well to historic data with the manual RF assay, thus pH 12.4 was utilised for further experiments until the variability studies when the suitability of an alternative pH was re-considered.



7.4.3 Assay Sensitivity and Method Equivalence

The sensitivity of the assay was tested by evaluating the least amount of chemically degraded sample that could be measured, as in the prior chapter on development of the RF assay using pure plasmid samples. Samples were subjected to prolonged treatment, as described in the Methods and Materials - Chapter 2, for 0, 2, 4, 6, 9, 12, 18, 24, 28, 31 and 48 hours. These samples were identical to those analysed previously in using the manual RF assay technique. The degraded samples were analysed using agarose gel electrophoresis and concurrently assayed using the PicoGreen fluorescent dye with the Multiprobe Ex II™ liquid handling device.



Analyses of the samples using agarose gel electrophoresis confirmed the progressive degradation of samples using the chemical technique, Figure 7.8. This progressive degradation is identified with the decreasing amounts of supercoiled DNA and increasing amounts of open-circular and linear forms of plasmid DNA. Densitometric scanning of the gel, shown in Figure 7.8, showed supercoiled plasmid percentage content from 88% to 17%.

For fluorescence analysis using the Multiprobe Ex II™ chemically treated 6.9 kb plasmid samples were laid out as shown in Figure 7.9A below.

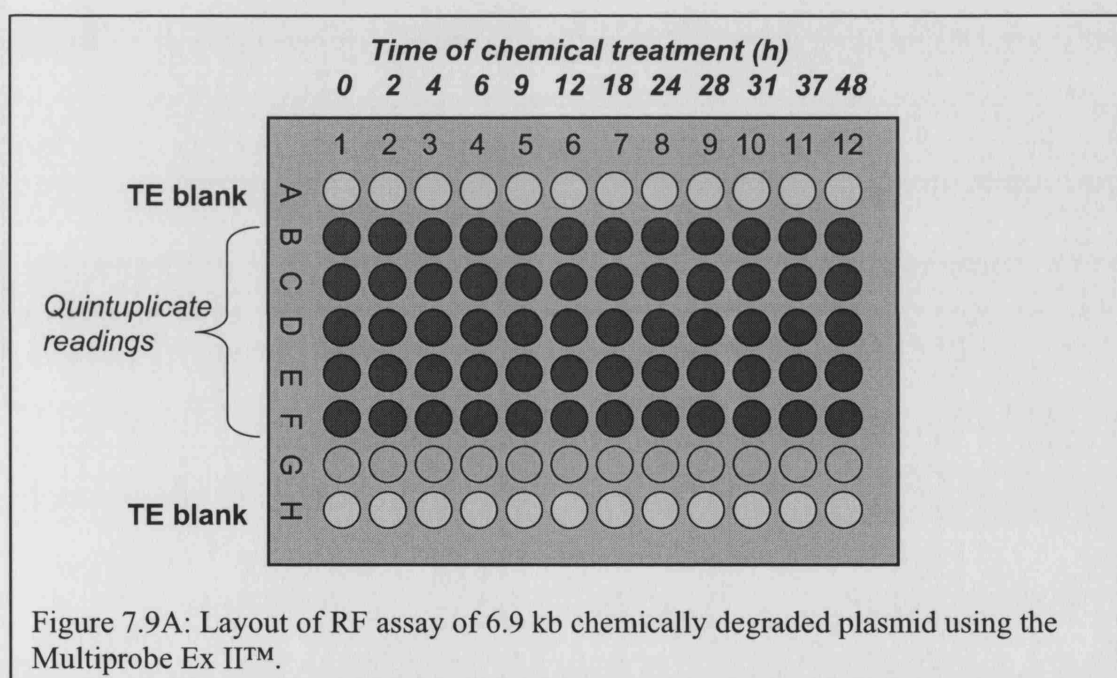


Figure 7.9B shows the result of RF analysis, the ratio was shown to fall from 0.594 (0h degradation) to 0.01 (48h degradation). Statistical differences could be discerned for all times of degradation tested above 6 hours by both agarose gel densitometry and RF assay. Correlation between the agarose gel densitometry and the RF assay was good with a coefficient of $R^2 = 0.96$.

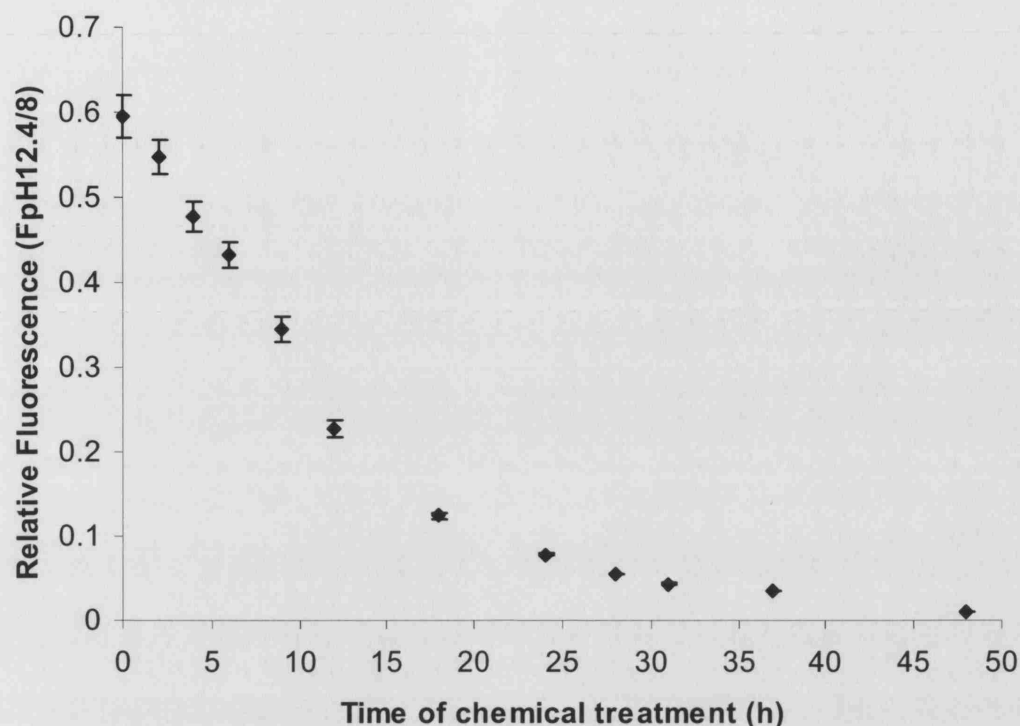


Figure 7.9B: Relative Fluorescence of 6.9kb plasmid subjected to chemical degradation from 0- 48 hours as described in Chapter 2. Plasmid concentration was 500 ng/mL. Error bars show the standard deviation of quintuplicate samples.

The equivalence of method for the 6.9 kb plasmid was shown through the direct comparison of fluorescence results using the manual assay and the robot based assay for the identical chemically treated samples. Figure 7.9C shows the direct comparison of the two samples and correlation. As correlation was very good, with a coefficient of $R^2 = 0.97$, and the line of linear regression almost intercepts the axes at 0, the two methods can be demonstrated to be equivalent.

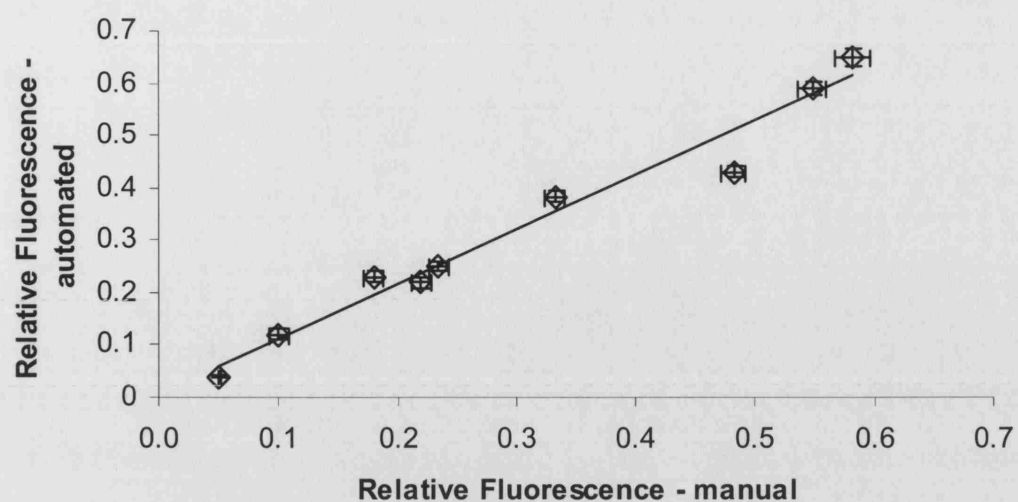
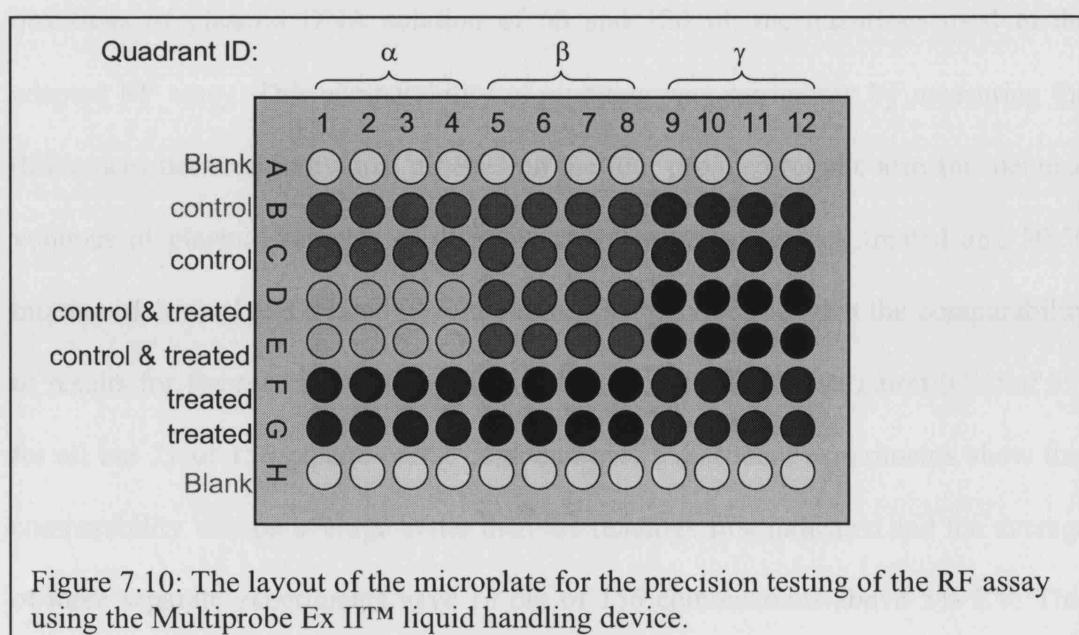


Figure 7.9 C: Correlation of Relative Fluorescence for 6.9kb plasmid attained using the manual assay and the automated assay. Plasmid concentration was 500 ng/mL. Relative Fluorescence assay was conducted at pH 12.4. Error bars represent standard deviation of triplicate data. $R^2 = 0.97$.

7.4.4 RF Assay precision and variability using the automated liquid handling system

The Multiprobe Ex II™ robotic liquid handling system was set up according to the algorithm through the WinPREPT™ programme. The workstation was integrated with the FluoroCount microplate reader. Prior to starting any work, each operation was observed visually with blank samples to ensure that it performed to the instructed specifications of the algorithm. Air bubbles were removed from the tubing of the system so that error in the aspiration of the liquid could be minimised. In a previous study on the usage of the robotic system with ultra-pure water, the variability of the dispersion volume was below 2% CV (coefficient of variation), (Ahrweiler et al., 2002).

Degraded samples of 6.9 kb plasmid were prepared using the chemical treatment cited in the “Methods” of Chapter 2. The treatment was carried out to yield samples that were mostly open-circular in nature as confirmed by agarose gel electrophoresis. A 50:50 mixture of control and degraded 6.9kb mixture was created. Samples of control, degraded and the 50:50 mixture of concentration of 500 ng/mL



were then used to determine assay precision for plasmid streams of differing characteristics. The layout of the microplate is shown in Figure 7.10.

The fluorescence enhancement of the PicoGreen- DNA complexes is dependent on the DNA conformations whereby linear and open-circular plasmid forms yield greater signals than supercoiled molecules. Fluorescence signals were read before and after the addition of alkali to pH 12.4. Measurements were corrected for background fluorescence using the readings of “blank” cells containing only TE buffer and PicoGreen reagent.

The automated RF assay was validated with respect to:

- Inter-tip precision and comparability (between tips 1-4)
- Inter-row and inter- column comparability for adapted RF assay
- Inter-quadrant comparability (between quadrant α , β and γ)
- Intra-assay precision (between control, control & treated, and treated samples)

7.4.4.1 Inter-tip Comparability

The comparability of the pipetting of the Multiprobe Ex II™ was evaluated for quantities of plasmid DNA solution of 60 and 120 μ l, the quantities used in the adapted RF assay. This comparability of pipetting was carried out by measuring the differences between individual pipettes on the four pronged robotic arm for identical volumes of plasmid samples of differing characteristics (control, treated and 50:50 mixture of control and treated). From Table 7.3 it may be seen that the comparability of results for fluorescence readings was below a coefficient of variation (CV) of 5% for all but 21 of 156 pipette prong combinations. Subsequent experiments show that comparability was on average better than the readings first indicated and the average of three separate experiments gave 13 out of 156 combinations above 5% CV. This

indicates that the Multiprobe Ex II™ liquid handling device is able to precisely pipette the volumes necessary for the execution of the RF assay in conjunction with the integrated microplate reader with acceptable variation. This variation is thought to be due to bubbles and this may be expected to decrease as learning effects would result in the greater elimination of trapped air within the pipes. The variation should

Between lanes:	1&2	1&3	1&4	2&3	2&4	3&4	9&10	9&11	9&12
control DNA	2.51	1.41	2.95	1.11	5.46	4.36	1.17	0.86	1.45
control DNA	4.15	8.96	6.04	4.82	1.89	3.21	3.63	2.96	4.45
control and treated DNA	1.41	5.25	2.54	3.84	1.14	2.92	3.29	5.14	6.12
control and treated DNA	2.1	0.3	3.06	2.4	0.96	3.55	0.5	6.13	4.62
treated DNA	1.2	2.67	0.94	1.47	0.26	1.38	0.18	2.77	0.6
treated DNA	1.44	1.37	0.27	2.81	1.71	0.8	3.64	0.19	0.25
Between lanes:	5&6	5&7	5&8	6&7	6&8	7&8	10&11	10&12	11&12
control DNA	1.83	0.49	5.87	1.34	7.69	6.36	2.03	0.29	2.32
control DNA	3.79	4.8	10.1	1.01	6.32	5.32	0.66	0.83	1.49
control and treated DNA	3.22	3.88	0.4	7.09	3.62	3.48	1.85	2.83	0.98
control and treated DNA	5.84	2.75	4.2	3.1	1.64	1.45	5.63	4.13	1.5
treated DNA	1.17	0.14	0.28	1.31	0.89	0.42	2.59	0.77	3.36
treated DNA	0.2	1.34	0.41	1.14	0.21	0.93	3.45	3.39	0.06
Between lanes:	1&5	2&6	3&7	4&8	5&9	6&10	7&11	8&12	
control DNA	1.82	1.14	0.91	1.09	4	4.66	5.36	3.32	
control DNA	3.69	3.32	0.49	7.76	5.85	1.57	1.92	8.71	
control and treated DNA	1.49	3.13	0.12	0.65	2	4.5	0.75	3.71	
control and treated DNA	4.27	3.67	1.82	2.99	3.48	1.86	6.86	3.91	
treated DNA	0.01	0.03	2.82	0.67	1.49	2.84	4.11	1.17	
treated DNA	1.48	2.73	1.22	0.81	3.65	0.19	2.12	2.99	

Key: % Coefficient of Variation

	< 5 %
	5 – 7 %
	7 – 10 %
	> 10 %

Table 7.3: Inter-tip comparability of the Multiprobe Ex II™ liquid handling device.

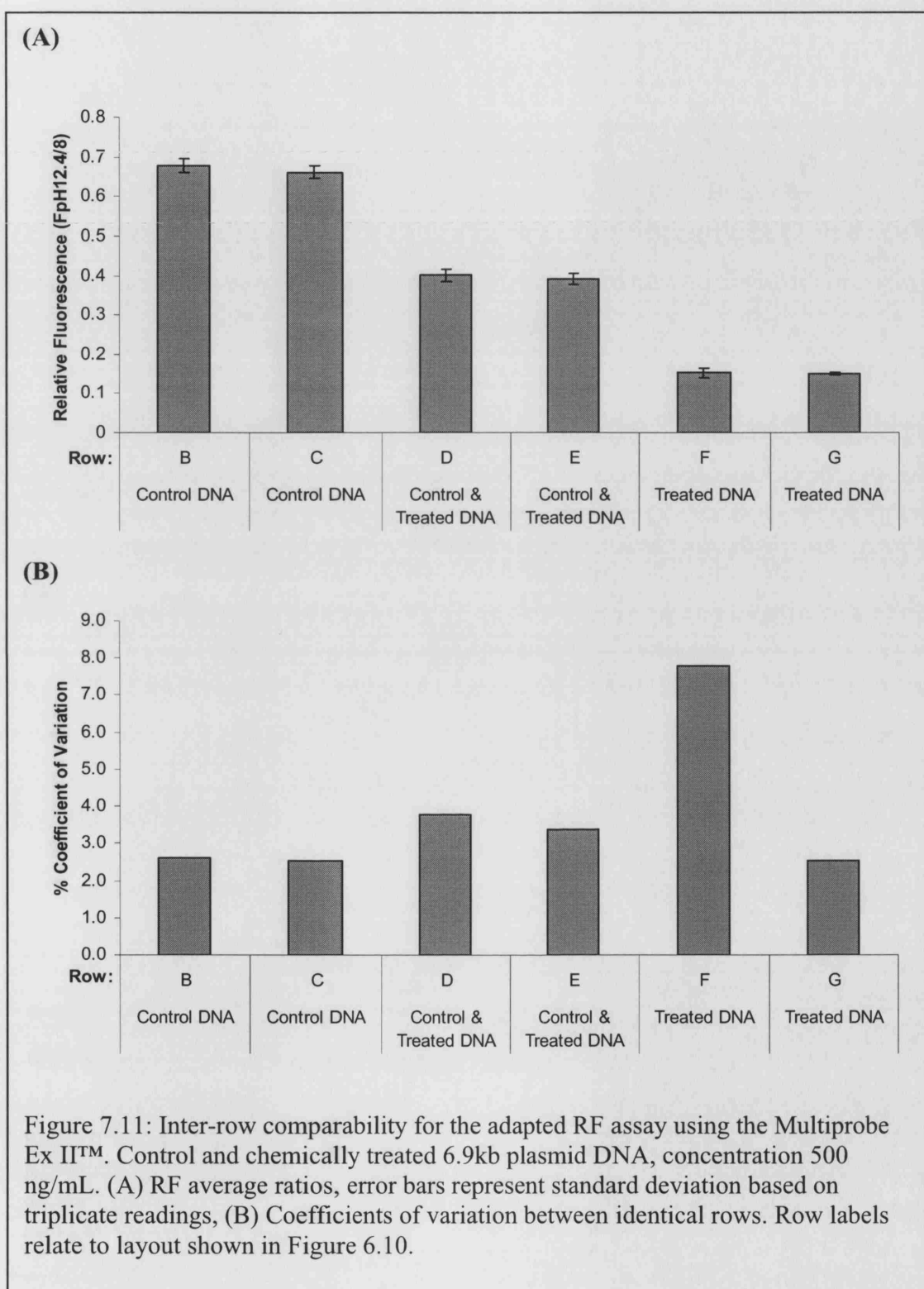
decrease to levels approaching the manufacturer's expected comparability and precision of all tip combinations within 5% CV.

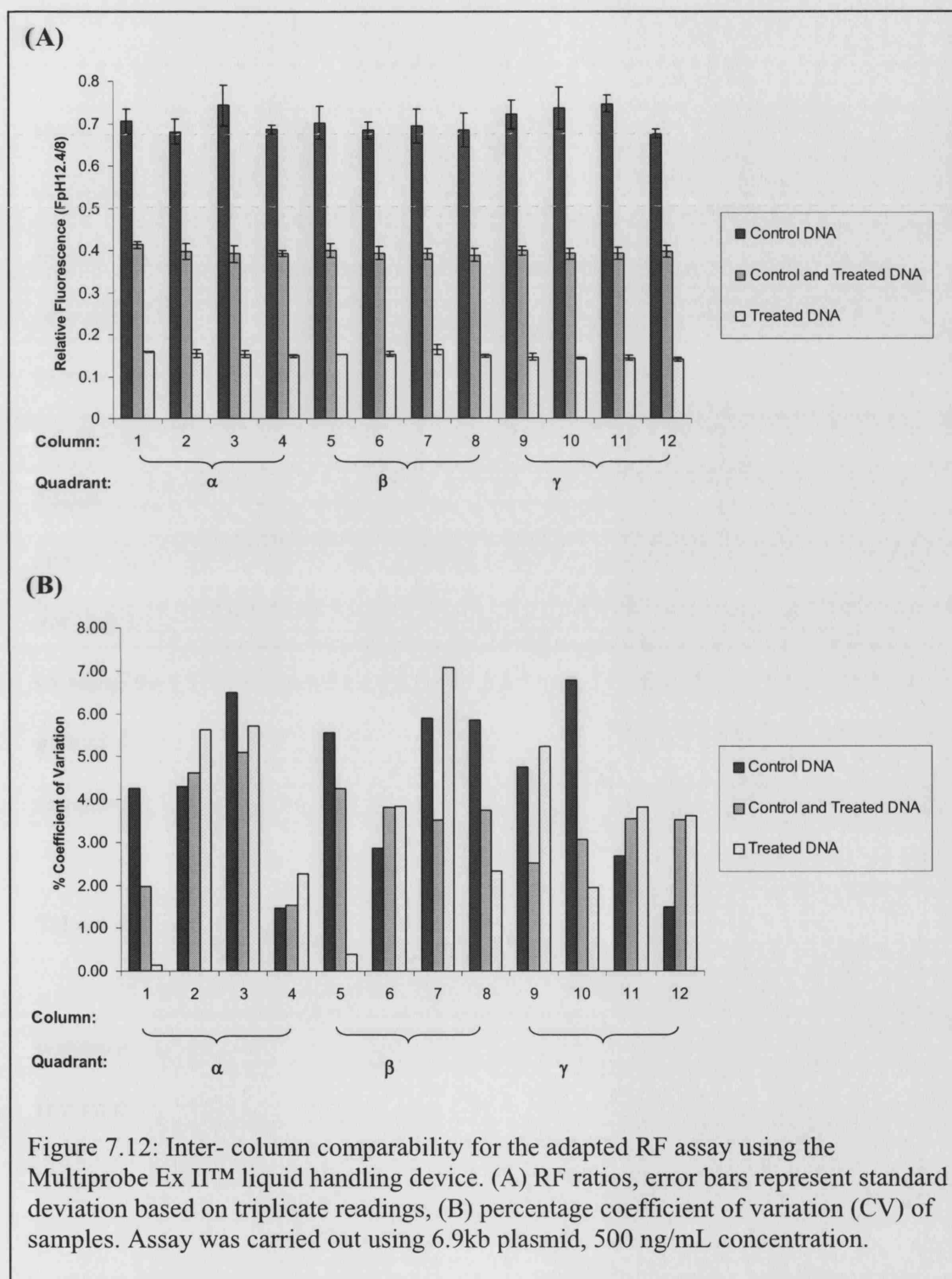
The raw fluorescence readings for the control and treated samples showed that under standard conditions control samples yielded lower fluorescence values than the 50:50 mixtures and the treated samples. This agrees with previous findings that fluorescence enhancement of PicoGreen-DNA complex was dependant on conformation of DNA (Levy et al., 2000b; Singer et al., 1996) and also agrees with findings described in Chapter 5 on the development of the manual RF assay. Fluorescence signals also corresponded well to historical data from the manual assay confirming the equivalence of the manual and automated RF assays. After the addition of alkali, RF ratio values also corresponded well to historical data and the comparability of the pipette prongs was 18 out of 156 combinations above a 5% CV as an average of three experiments.

7.4.4.2 Inter-row and inter-column comparability

The adapted RF assay results were then analysed to establish that rows were comparable when dispensed by the Multiprobe Ex II™. It was found that RF results were comparable between identical rows, the highest variation being 7.72 % CV as shown in Figure 7.11.

When the RF results were compared for the differing columns on the 96 well microplate, comparability was also found to be very strong, Figure 7.12A. Variability was found to range between 0.13 and 6.09 % CV when using the four pronged pipette arm.





7.4.4.3 Inter-quadrant comparability

Variations between the quadrants labelled in Figure 7.10 were then investigated for the adapted RF assay. As with the inter-row and inter-column comparability, this assesses whether the position of the sample on the plate has any impact on the performance of the assay. The 96 well microplates were filled by the four pipette arm of the Multiprobe Ex II™ from column 1- 12 and then from row A to H. For the 6.9 kb plasmid there was good comparability of the assay results as shown in Figure 7.13A. The coefficient of variation ranged from 0.91 to 4.22%, Figure 7.13B. When the assay plan was changed so that the wells were filled from row A to H and then columns 1-12, there was also good comparability of results varying from 1.00% to 5.63% coefficient of variation. This indicates that the location of samples on the plate and the speed of pipetting has little impact on the results gained for the RF assay and thus results gained from samples on one part of the plate are directly comparable to results in other parts of the same microplate.

7.4.4.4 Intra-assay precision

When all data for the entire microplate is considered, it may be seen that comparability between the RF assays for control, treated and 50:50 mixture varied from 0.67 to 4.21 % coefficient of variation as shown in Table 7.4.

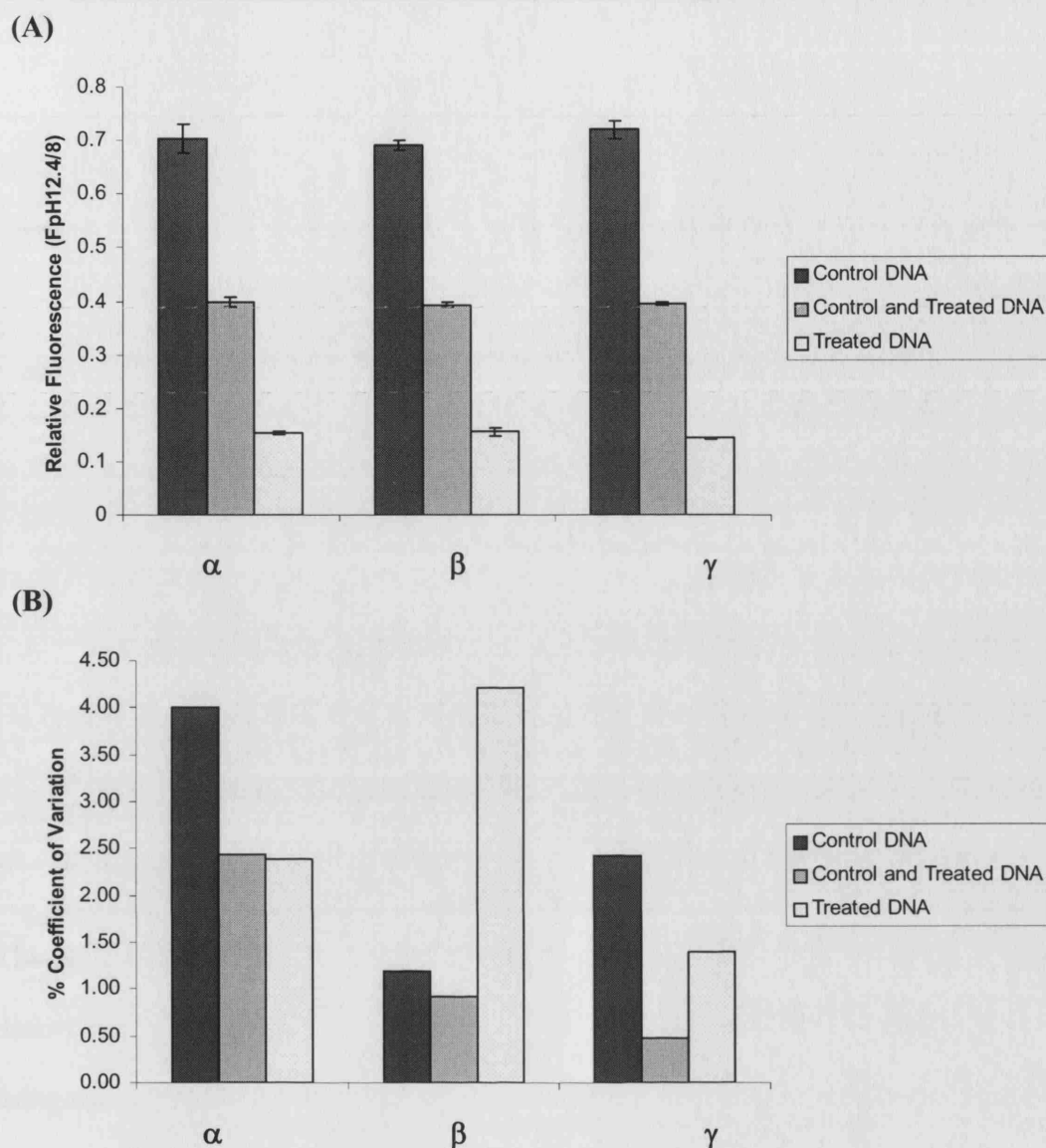


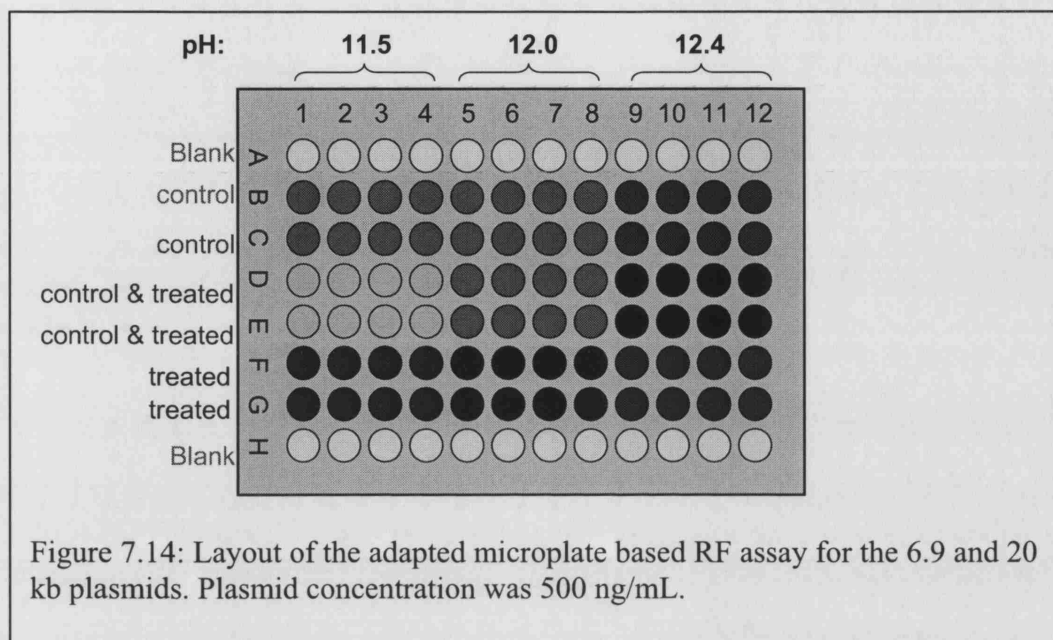
Figure 7.13: Inter-quadrant (α , β , γ) comparability for the adapted RF assay using 6.9 kb plasmid, concentration 500 ng/mL. (A) RF average ratios, error bars represent standard deviation based on triplicate readings. (B) percentage Coefficient of variation. Quadrant labels relate to layout in Figure 7.10.

	Average	Standard Deviation	% CV
Control DNA	0.71	0.015	2.08
Control and Treated DNA	0.40	0.003	0.67
Treated DNA	0.15	0.006	4.21

Table 7.4: Intra-assay comparability for the RF assay for control, treated and 50:50 mixture of 6.9kb plasmid DNA solution. Plasmid concentration was 500 ng/mL. Results are from triplicate microplate data.

7.4.4.5 RF assay variability using the 20 kb plasmid

Samples of control, treated and 50:50 mixture were then subjected to the RF assay under alkali conditions of pH 11.5, 12.0 and 12.4 as shown in Figure 7.14.



The pH was varied in order to assess whether the alkali strength (pH 12.4) previously determined as ideal for the 6.9 kb plasmid was still suitable for the 20 kb plasmid using the adapted assay. This was a particular concern for 20 kb plasmids subjected to varying degrees of determination as this may have lead to a loss of precision, reliability and ruggedness for the assay.

The RF assay was conducted under these conditions for three replicates of 20 kb plasmid samples and the results analysed with respect to inter-tip comparability, inter- row comparability and intra-assay precision.

As with historic data from the manual assay, there was no significant denaturation seen at pH 11.5 for all types of the 20kb plasmid solution. Before the addition of alkali, the variation with the pipetting of the sample solutions alone was found to be below 5% CV. Thus dispensation volumes were found to be precise and

comparable to the manufacturer's specifications. After the addition of alkali the inter-tip variation was calculated for the entire assay by assessing the coefficient of variation for the permutation of tips in the dispensation of plasmid solution, PicoGreen, TE buffer and NaOH into the 96 well microplate, this variation was found to be below 10% across the entire microplate. In general, analysis of the RF assay at pH 12.0 and 12.4, the variation was below 6.5% as shown in Table 7.5. This was a good indication that even at pH 12.4 the RF assay was precise and robust with reference to the 20 kb plasmid.

20 kb Plasmid		pH 12.0		pH 12.4	
	Row	RF Average	% CV	RF Average	% CV
Control DNA	B	1.02	4.7	0.50	6.3
	C	0.95	1.7	0.48	3.2
Control and Treated DNA	D	0.74	3.5	0.24	3.7
	E	0.68	2.6	0.22	4.7
Treated DNA	F	0.53	3.6	0.09	4.1
	G	0.53	4.2	0.08	2.8

Table 7.5: Average RF ratios and coefficient of variation for the adapted RF assay using 20kb plasmid. Plasmid concentration was 500 ng mL⁻¹. Results are based on quadruplicate data.

In inter-row evaluation the variations were all found to be below 10%. For the intra-assay evaluation the percentage CV was found to be between 3.6 and 5.8% as shown in Table 7.6. Thus, the adapted RF assay was found to be precise and robust with respect to the 20 kb plasmid.

% Coefficient of Variation				
20 kb Plasmid	pH 12.0		pH 12.4	
	RF Average	% CV	RF Average	% CV
Control DNA	0.985	5.76	0.492	5.10
Control and Treated DNA	0.709	5.17	0.228	5.43
Treated DNA	0.528	3.67	0.084	3.55

Table 7.6: Average Relative Fluorescence (RF) ratios and Intra-assay variation for the adapted RF assay using 20kb plasmid. Plasmid concentration was 500 ng mL⁻¹.

7.5 Conclusions

The RF assay has been successfully adapted for use with the Multiprobe Ex II™ liquid handling system. Assay results for the 6.9 and 20 kb plasmids were found to be comparable to results gained from manual operation and variability of response was also found to be comparable with manual operation.

The greatest impact of the automation of the assay is that the use of the liquid handling system allows far greater sample throughput and the potential testing of a large number of process conditions. This makes the use of the automated assay applicable to current trends towards assay development and systems that allow the measurement of a large number of critical quality parameters and performance attributes in plasmid process streams. These process analytic techniques allow the continuous quality control of process streams and hence decrease the likelihood of inefficiencies, low quality product and wasted time and expense. Thus, the use of liquid handling systems in conjunction with assays may become an integral part of the validation of plasmid process streams in the future; whereby assays may be rapidly performed at or near the product line decreasing the time delay seen with off-site testing and allowing the addressing of process issues in a timely and efficient manner.

The area of micro-biochemical engineering represents a new paradigm in the development of rapid, economic plasmid production processes. This has particular significance in the light of current government emphasis on the containment of healthcare costs and the improvement of the speed of medicines to market. The use of micro-plates in combination with liquid handling systems allow the exploration of a large range of processing environments from small-scale fermentation through to the downstream purification processes. The automated micro-plate based assay represents

an analytic technique that is suitable for use in micro-biochemical engineering and combines flexibility of operation with ease of use.

8 CONCLUSIONS AND FUTURE WORK

The characterisation of complex biologics is vital for enhanced bioprocess understanding, cost effective design, development and establishment of process analytical technologies. In particular, an understanding of the response to hydrodynamic stresses of the biological product and its process contaminants provides important information for the scale-up of plasmid production and the optimisation of the quality of plasmid pharmaceutical products.

Current pharmaceutical production is faced with the key challenges of long development time, low success rate of new compounds and the high cost of development and marketing of new drugs, as discussed in Chapter 1. Additionally, although medicines are becoming more complex and healthcare markets further segmented, there is an emphasis on decreasing associated costs and increasing the speed of drugs to market. Therefore, current focus in drug development is on speed and the economical use of product for process stream characterisation and validation. The use of ultra scaled-down equipment, bioprocess modelling and process analytical techniques all address this necessity for rapid and efficient drug development.

This project has sought to address the challenges posed to biochemical engineers seeking to optimise plasmid recovery at key unit operations during plasmid processing. In Chapter 3, bacterial cells were found to be detrimentally affected by increases in agitation and airflow rate during fermentation which resulted in premature cell lysis. Further studies in this area should assess the significance of this observation by quantifying the effect of a range of airflow rates and agitation speeds on plasmid quality, yield and contaminant levels at the pilot plant scale. In addition, the use of PicoGreen as a process analytic technique for the direct measurement of spent broth and the TE wash of cell pastes should be explored.

In Chapter 4, the use of the pilot plant scale continuous centrifugation on bacterial cells was found to be the cause of increased levels of host cell chromosomal contamination in the process stream. As fragmented particles of chromosomal DNA may purify with the plasmid product, there are important implications for the quality of the plasmid product. Direct measurement of released DNA at the cell re-suspension stage may also provide a simple and fast technique to judge the product quality before further downstream work is carried out. This allows the plasmid production process to be more addressable in terms of utilising current process knowledge to optimise product yield. The suitability of this method for plasmid streams should be further established and validation carried out.

In Chapter 5, a simple and rapid microplate-based technique for the testing of plasmid integrity was established and validated. The method was shown to be applicable to a wide variety of DNA sequences and sizes and was used successfully with bacterial artificial chromosomes. This assay has been used successfully by other researchers within the Biochemical Engineering Department of University College London to assess the effect of engineering environments such as those within valves and pumps on shear labile plasmids. This assay was then successfully adapted for use with a robotic liquid handling system (Chapter 7) and proved to be a fast, reproducible and efficient qualitative technique that may be used within an industrial setting. Future work in this area may see the further application of this assay to the characterisation of shear forces exhibited within key units of operation and may eventually lead to the improved design of equipment for use with shear labile plasmids. In the short term, characterisation of the shear forces endured by plasmids may lead to improved equipment selection. Further work on the assay should include determination of the sensitivity with calibrated breaks in plasmid DNA produced by

enzymatic means. The RF assay may also be extended to use as an abasic site assay with the use of DNA N-glycosylases. Additional parameters of interest for the development of inexpensive rapid assays include the secondary structure of DNA, which is currently monitored using spectroscopic methods such as Fourier-transform infrared spectroscopy.

It has been argued that in the future a combination of microchip-based mutation detection devices combined with an array of chromatographic, electrophoretic, hydrodynamic, and spectroscopic methods can be employed to rigorously establish the properties of identity and biological potency of plasmid DNA drugs, Middaugh et al., 1998. This would help drug manufacturers to face the key challenges discussed earlier by reducing cost and time to market through more rapid and effective process validation and quality assurance.

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APPENDIX 1

**An automated microplate-based method for monitoring DNA strand breaks in plasmids
and bacterial artificial chromosomes.**

The following paper is based largely work described in Chapter 5.

APPENDIX 2

Large-scale plasmid DNA processing for vaccine and gene therapy: impact of centrifugation and resuspension of bacterial cells.

The following paper has been submitted for journal publication and is based largely on Chapter 4.

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Figure Legends

Figure 1. (A) Fermentation kinetics: dissolved oxygen tension (% DOT) for 75 (◆) L and 450 (■) L cultures, and optical density (OD 600 nm) for 75 (◆) L and 450 (■) L cultures. (B) Plasmid yield (□) and relative plasmid integrity (□) throughout the 450L fermentation. Plasmid yield and relative plasmid integrity were determined as detailed in Methods and Materials.

Figure 2. Effect of centrifuge design and operating conditions. Agarose gel electrophoresis of isopropanol precipitated pQR150 samples derived from cells harvested using (A) a disk stack centrifuge operated at 50 (lane 1) and 250 Lh⁻¹ (lane 2) and (B) tubular bowl centrifuge operated at 30 (lane 1) and 60 Lh⁻¹ (lane 2). S1 is a Qiagen purified pQR150 plasmid sample derived from laboratory scale culture, S2 is S1 subjected to 60 °C for 24 h to accelerate conversion of supercoiled circular (SC) pQR150 into open circular (OC) and linear forms. M is the DNA marker λ HindIII. A 0.8% agarose gel was run for 10 h at 40V; the gel was deliberately overloaded to enhance visualisation of low molecular weight species.

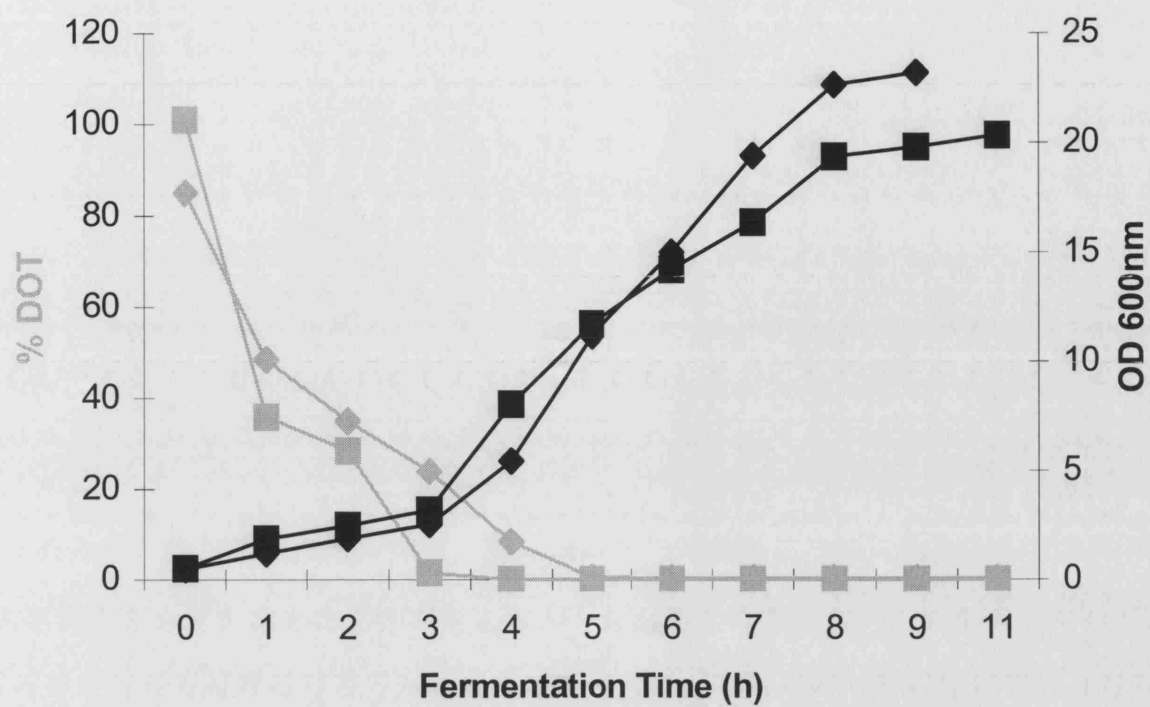
Figure 3. Effect of lysis scale and clarification procedure. Agarose gel electrophoresis of isopropanol precipitated pQR150 samples derived from cells harvested using (A) tubular bowl centrifuge operated at 30 (lane 1, 3, 5, 7) or 60 Lh⁻¹ (lane 2, 4, 6, 8); lysed at 0.5 ml scale (lane 1-4) or 300 ml scale (lanes 5-8); the floc was removed by centrifugation (lanes 5-6) or filtration (lanes 7-8), some 0.5 ml scale preparations were incubated with alkali and detergent for 1 hour (1 h) before neutralisation (lanes 3-4) and (B) disk stack centrifuge operated at 50 (lane 1, 3, 5, 7) or 250 Lh⁻¹ (lane 2, 4, 6, 8); lysed at 0.5 ml scale (lane 1-4) or 300 ml scale (lanes 5-8); the floc was removed

by centrifugation (lanes 5-6) or filtration (lanes 7-8), some 0.5 ml scale preparations were incubated with alkali and detergent for 1 hour (1 h) before neutralisation (lanes 3-4). (C) Isopropanol precipitated DNA extracted from DH5 α pQR150 cells harvested with the disk stack centrifuge operated at 50 (lane 1) or 250 Lh⁻¹ and host cell containing no plasmid (lane 3). Chr. is chromosomal DNA, other abbreviations are as seen in Fig. 2. Gels were 0.8% agarose and were run for 12 h at 40V (A- B) or 3 h at 80V (C).

Figure 4. Characterisation of bacterial cells. Analysis of released (A) proteins and (B- C) nucleic acids from bacterial cells harvested as described in Fig. 3 and resuspended in TE buffer at 4 °C for 30 minutes. After resuspension, cells were centrifuged briefly and supernatants analysed. Nucleic acids were determined by (B) PicoGreen fluorescence enhancement and (C) Agarose gel electrophoresis. Abbreviations are same as in Fig. 3. Gel was 0.8% agarose and run for 12 h at 40 V.

Figure 5. Effect of cell resuspension conditions. (A) Kinetics of nucleic acids release from cells harvested using a tubular bowl centrifuge operated at 60 Lh⁻¹ and resuspended at 4 °C, (.....◇.....) 13 (.....□.....) and 25 (—△—) °C. After resuspension cells were centrifuged briefly and supernatant analysed using PicoGreen, dilution of supernatant was 1 /5000 and (B) agarose gel electrophoresis of Qiagen purified DNA extracted from cells resuspended at 13 °C for 0-6 h. Abbreviations are same as in Fig. 3.

A



B

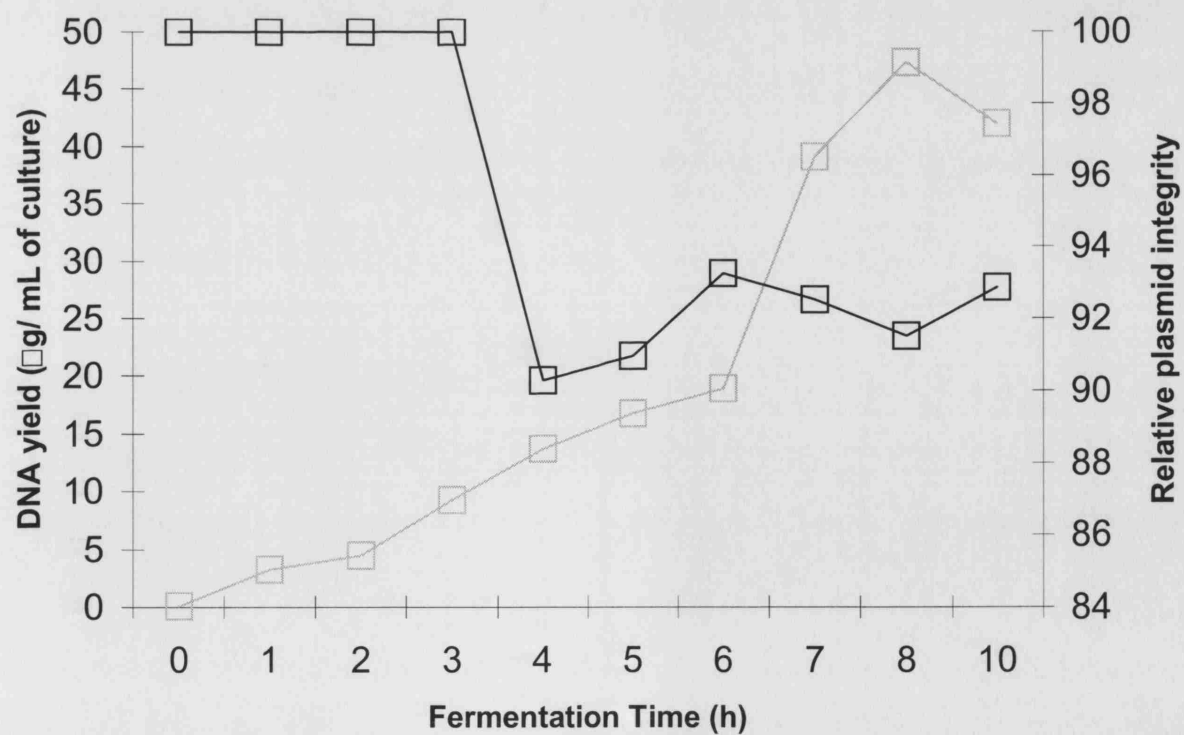
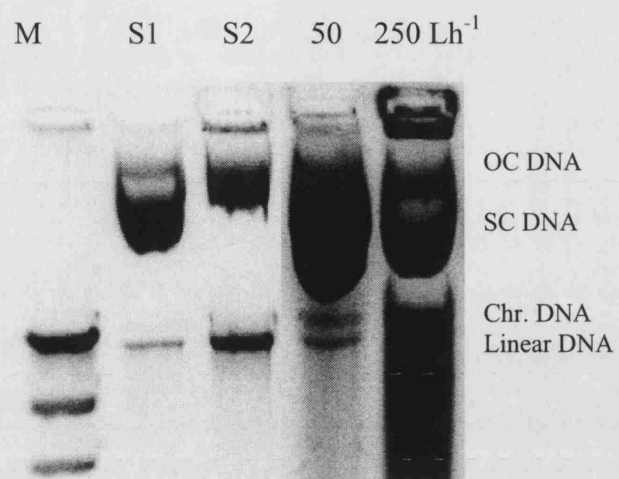
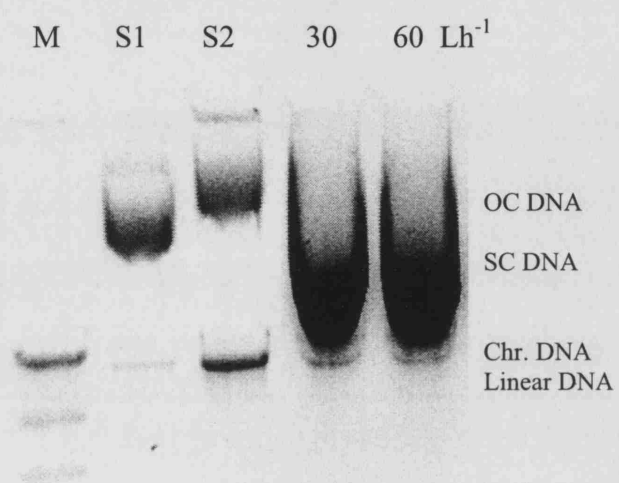


Figure 1

A**B**

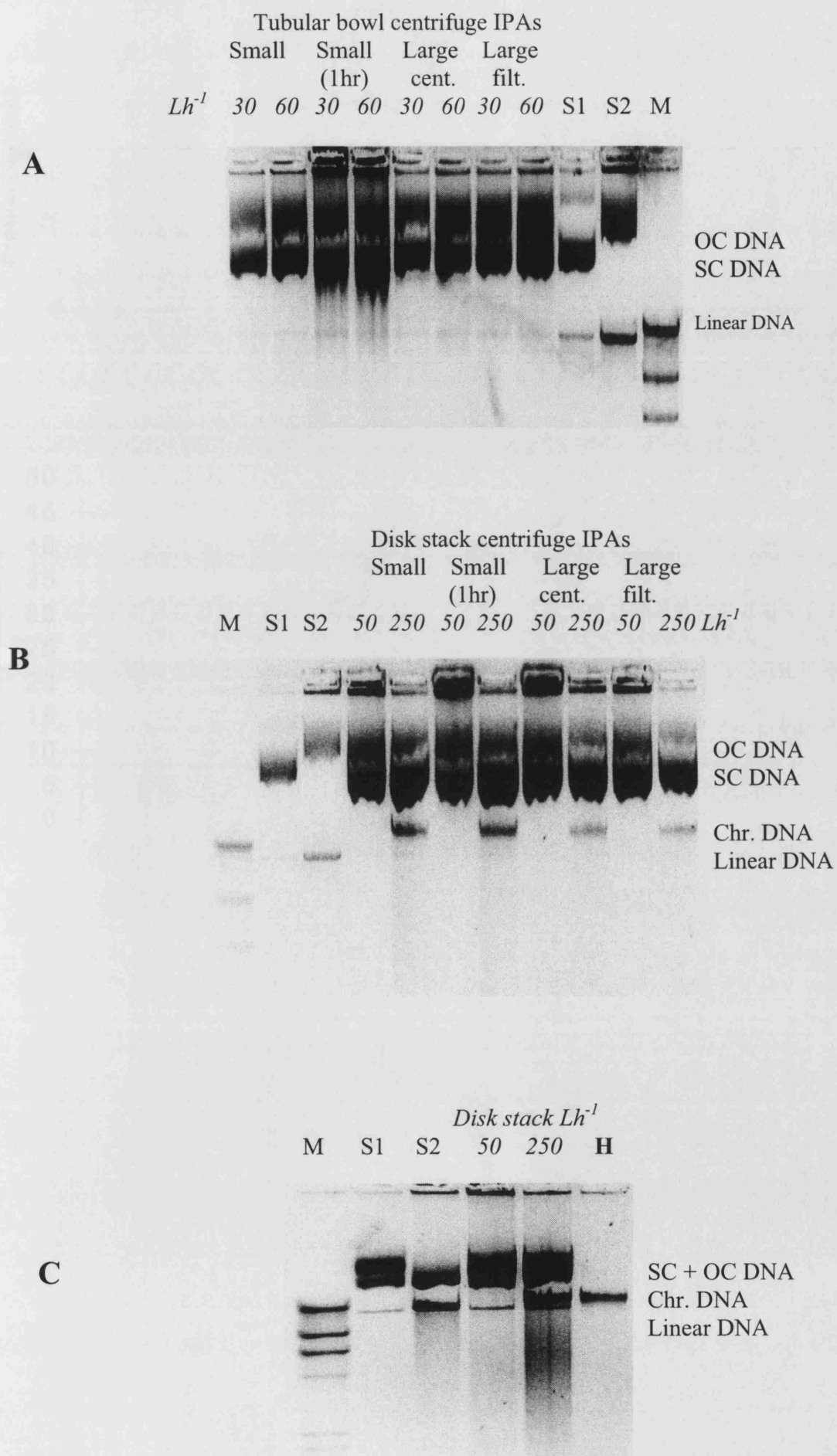


Figure 3

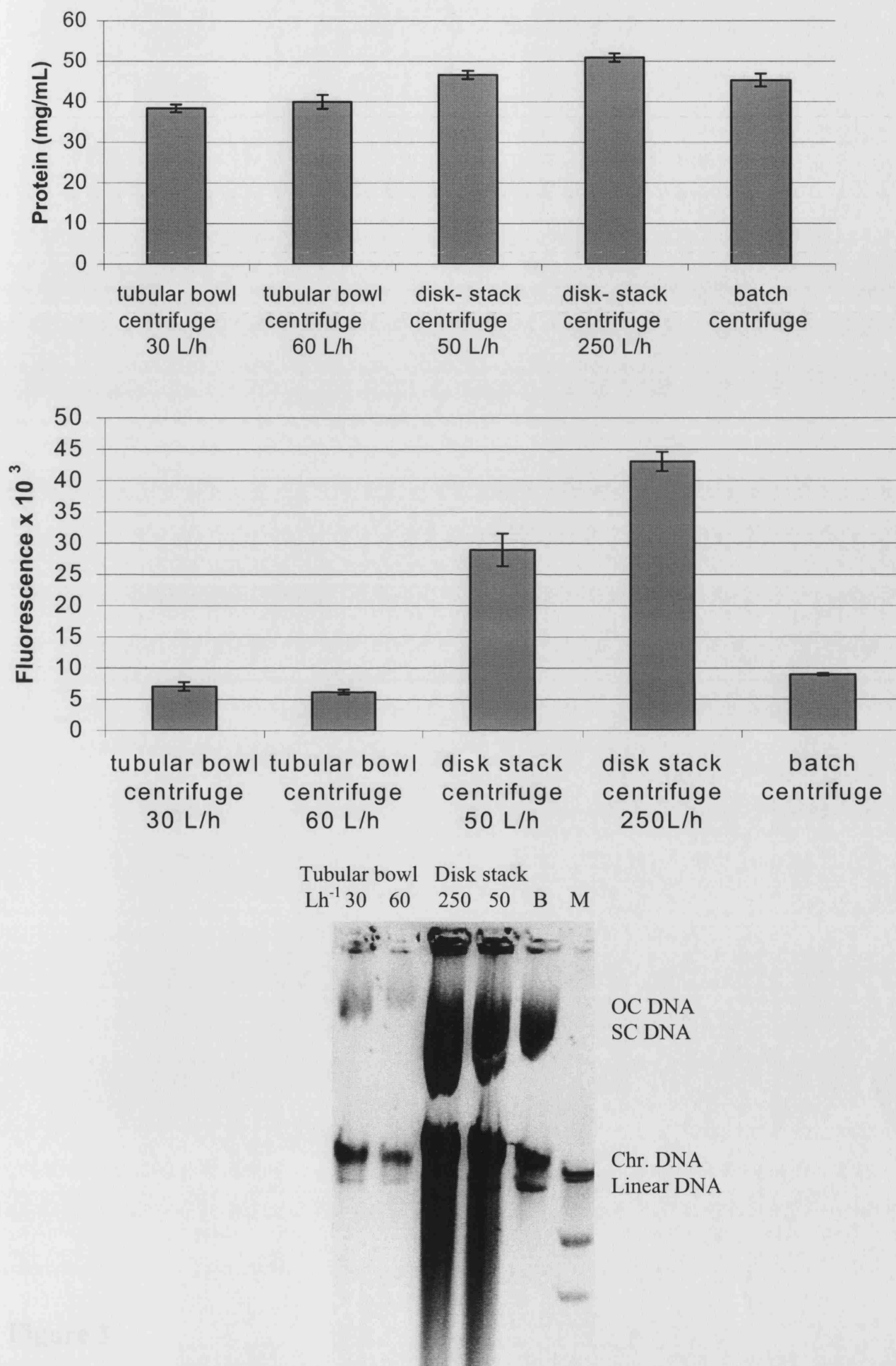


figure 4

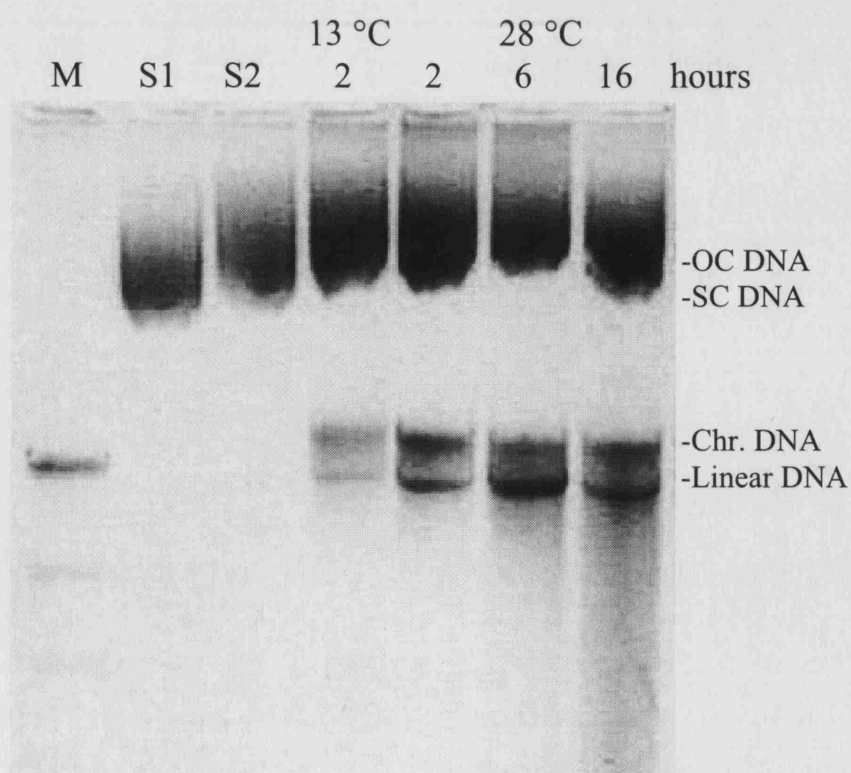
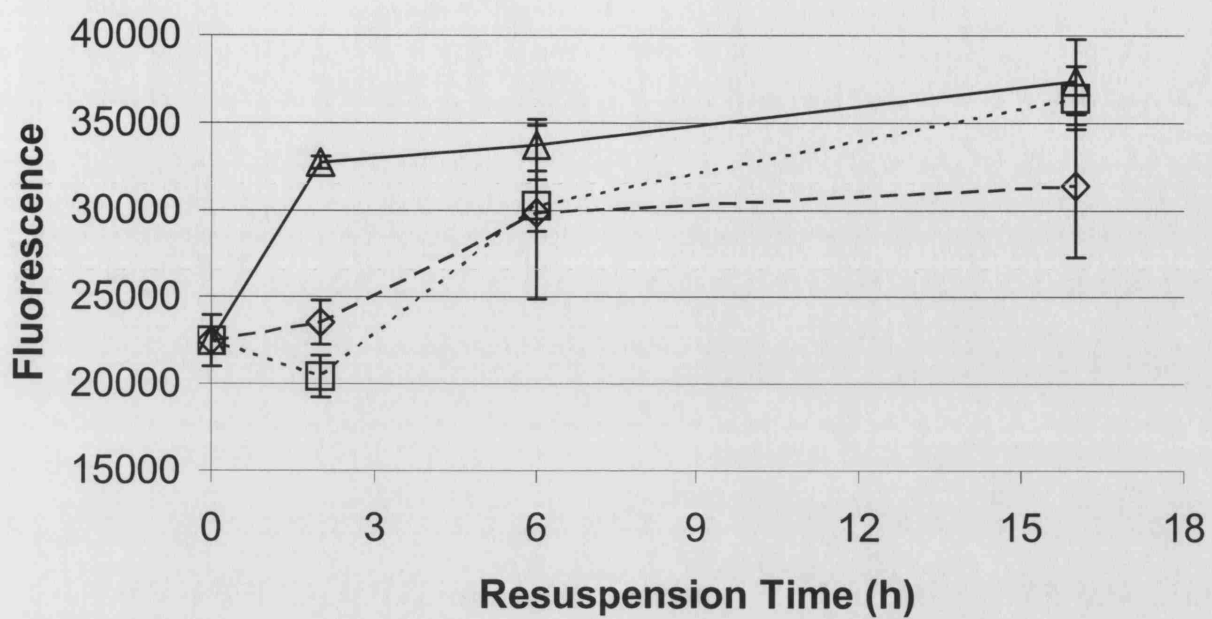


Figure 5

APPENDIX 3

Qiagen plasmid purification protocols

(Mini, Midi/Maxi, Mega/Giga)

Qiagen kits and protocols were used for all production of ultrapure plasmid DNA except for the production of BAC DNA where the protocol used in Wade- Martins et al., 1999 was followed from the DNA extraction to the production of isopropanol precipitates. In order to produce ultrapure BAC DNA, isopropanol precipitates were resuspended in TE buffer and loaded onto Qiagen Maxi or Mega columns and the procedure detailed below for the Maxi and Mega kits were followed.

QIAGEN Plasmid Mini Handbook 10/2001 10 Protocol

Plasmid Mini Purification Protocol

This protocol is for Mini (up to 20 µg) preparations of high-copy plasmid DNA from cultures of *E. coli*.

Things to do before starting

- Add the provided RNase A solution to Buffer P1 before use (spin down RNase A briefly before use). Buffer P1 should then be stored at 2–8°C and is stable for 6 months.
- Check Buffer P2 for SDS precipitation due to low storage temperatures. If necessary, dissolve the SDS by warming to 37°C.

- Chill Buffer P3 at 4°C.
- Optional: To confirm purification or to identify a problem, samples may be taken at specific steps for analysis on an agarose gel. Appropriate samples and volumes are indicated in the protocol below.

Procedure

1. Resuspend the bacterial pellet in 0.3 ml of Buffer P1.

Ensure that RNase A has been added to Buffer P1. The bacteria should be resuspended completely, leaving no cell clumps.

2. Add 0.3 ml of Buffer P2, mix gently, and incubate at room temperature for 5 min.

After addition of Buffer P2, the solution should be mixed gently, but thoroughly, by inverting the tube 4–6 times. Do not vortex, as this will result in shearing of genomic DNA. The lysate should appear viscous. Do not allow the lysis reaction to proceed for more than 5 min. After use, the bottle containing Buffer P2 should be closed immediately to avoid any reaction between the NaOH and CO₂ in the air. If the buffer is left open for any length of time, it should be prepared fresh from stock solutions.

3. Add 0.3 ml of chilled Buffer P3, mix immediately but gently, and incubate on ice for 5 min. Precipitation is enhanced by using chilled Buffer P3 and incubating on ice. After addition of Buffer P3, the solution becomes cloudy and very viscous. To avoid localized potassium dodecyl sulfate precipitation, mix the solution gently, but thoroughly, immediately after addition of Buffer P3. Mix by inverting the tube 4–6 times.

4. Centrifuge at maximum speed in a microcentrifuge for 10 min. Remove supernatant promptly. Before loading the centrifuge, the sample should be mixed again. Centrifugation should be performed at maximum speed in 1.5 ml or 2 ml

microcentrifuge tubes (e.g., 10,000–13,000 rpm in a microcentrifuge). Maximum speed corresponds to 14,000–18,000 x g for most microcentrifuges. After centrifugation, the supernatant should be clear. If the supernatant is not clear, a second, shorter centrifugation should be carried out to avoid applying any suspended or particulate material to the column. Suspended material (which causes the sample to appear turbid) will clog the column and reduce or eliminate flow.

- Remove a 50 µl sample from the cleared lysate and save it for an analytical gel (sample 1).

5. Equilibrate a QIAGEN-tip 20 by applying 1 ml Buffer QBT, and allow the column to empty by gravity flow. Place QIAGEN-tips into a QIArack over the waste tray or use the tip holders provided with each kit (see “Setup of QIAGEN-tips” page 9). Flow of buffer will begin automatically by reduction in surface tension due to the presence of detergent in the equilibration buffer. Allow the QIAGEN-tip to drain completely. The resin bed will retain some buffer and will not readily dry out. QIAGEN-tips can therefore be left unattended. Do not force out the remaining buffer.

6. Apply the supernatant from step 4 to the QIAGEN-tip 20 and allow it to enter the resin by gravity flow.

The supernatant should be loaded onto the QIAGEN-tip promptly. If it is left too long and becomes cloudy due to further precipitation of protein, it must be recentrifuged before loading to prevent clogging of the QIAGEN-tip.

- Remove a 50 µl sample of the flow-through and save for an analytical gel sample 2).

7. Wash the QIAGEN-tip 20 with 4 x 1 ml Buffer QC.

Allow Buffer QC to move through the QIAGEN-tip by gravity flow. The first 2 ml is sufficient to remove all contaminants in the majority of plasmid DNA preparations.

The second 2 ml ensures complete removal of contaminants, and will also ensure consistent results in sequencing. (The second 2 ml is particularly necessary when large culture volumes or bacterial strains producing large amounts of carbohydrate are used.) It is particularly important not to force out residual wash buffers. Traces of wash buffer will not affect the elution step.

- Remove a 50 µl sample of the combined wash fractions and save for an analytical gel (sample 3).

8. Elute DNA with 0.8 ml Buffer QF.

Place the upper part of a QIArack over the lower rack fitted with clean 1.5 ml or 2 ml microcentrifuge tubes and collect the samples in the tubes. Alternatively, use the tip holders provided. Flow begins when Buffer QF is added. Drain the QIAGEN-tip by allowing it to empty by gravity flow.

- Remove a 50 µl sample of the eluate and save for an analytical gel (sample 4).

9. Precipitate DNA with 0.7 volumes (0.56 ml per 0.8 ml of elution volume) of room-temperature isopropanol. Centrifuge immediately at =10,000 rpm for 30 min in a microcentrifuge, and carefully decant the supernatant. Precipitation of DNA with isopropanol should be carried out with all solutions equilibrated to room temperature in order to minimize salt precipitation. Isopropanol pellets have a glassy appearance and may be more difficult to see than the fluffy, salt-containing pellets that result from ethanol precipitation. Marking the outside of the tube before centrifugation allows the pellet to be easily located.

10. Wash DNA with 1 ml of 70% ethanol, air-dry for 5 min, and redissolve in a suitable volume of buffer. The DNA pellet should be washed briefly in 70% ethanol, and then recentrifuged. The 70% ethanol serves to remove precipitated salt, as well as to replace isopropanol with the more volatile ethanol, making the DNA easier to

redissolve. A second wash with room-temperature 70% ethanol may improve results in more sensitive applications, such as transfection. After careful and complete removal of ethanol, the pellet should be air-dried briefly (approximately 5 min) before redissolving in an appropriate volume of TE buffer. Overdrying the pellet will make the DNA difficult to redissolve. Resuspend the DNA pellet by rinsing the walls to recover all the DNA. Pipetting the DNA up and down to promote resuspension may cause shearing, and should be avoided. DNA dissolves best under slightly alkaline conditions; it does not easily dissolve in acidic buffers.

Determination of yield

To determine the yield, DNA concentration should be determined by both UV spectrophotometry and quantitative analysis on an agarose gel.

Analytical gel (optional): to analyze the purification procedure, precipitate samples 1–4 (from steps 4–8) with 35 μ l of isopropanol. Rinse the pellets with 70% ethanol, drain well, air-dry, and resuspend in 10 μ l of TE, pH 8.0. Use 2 μ l of each for analysis on a 1% agarose gel.

QIAGEN Plasmid Midi/Maxi

QIAGEN Plasmid Purification Handbook 08/2003 17

Procedure

1. Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 2–5 ml LB medium containing the appropriate selective antibiotic. Incubate for ~8 h at 37°C with vigorous shaking (~300 rpm). Use a tube or flask with a volume of at least 4 times the volume of the culture.

2. Dilute the starter culture 1/500 to 1/1000 into selective LB medium. For high-copy plasmids inoculate 25 ml or 100 ml medium. For low-copy plasmids, inoculate 100 ml or 500 ml medium. Grow at 37°C for 12–16 h with vigorous shaking (~300 rpm).

Use a flask or vessel with a volume of at least 4 times the volume of the culture. The culture should reach a cell density of approximately $3\text{--}4 \times 10^9$ cells per ml, which typically corresponds to a pellet wet weight of approximately 3 g/liter medium (see page 68).

3. Harvest the bacterial cells by centrifugation at 6000 x g for 15 min at 4°C. 6000 x g corresponds to 6000 rpm in Sorvall® GSA or GS3 or Beckman™ JA-10 rotors. Remove all traces of supernatant by inverting the open centrifuge tube until all medium has been drained. If you wish to stop the protocol and continue later, freeze the cell pellets at –20°C.

4. Resuspend the bacterial pellet in 4 ml or 10 ml Buffer P1. For efficient lysis it is important to use a vessel that is large enough to allow complete mixing of the lysis buffers. Ensure that RNase A has been added to Buffer P1. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain. 5. Add 4 ml or 10 ml Buffer P2, mix gently but thoroughly by inverting 4–6 times, and incubate at room temperature for 5 min. Do not vortex, as this will result in shearing of genomic DNA. The lysate should appear viscous. Do not allow the lysis reaction to proceed for more than 5 min. After use, the bottle containing Buffer P2 should be closed immediately to avoid acidification from CO₂ in the air.

6. Add 4 ml or 10 ml of chilled Buffer P3, mix immediately but gently by inverting 4–6 times, and incubate on ice for 15 min or 20 min. Precipitation is enhanced by using chilled Buffer P3 and incubating on ice. After addition of Buffer P3, a fluffy white material forms and the lysate becomes less viscous. The precipitated material contains

genomic DNA, proteins, cell debris, and SDS. The lysate should be mixed thoroughly to ensure even potassium dodecyl sulfate precipitation. If the mixture still appears viscous and brownish, more mixing is required to completely neutralize the solution.

7. Centrifuge at $\approx 20,000 \times g$ for 30 min at 4°C. Remove supernatant containing plasmid DNA promptly. Before loading the centrifuge, the sample should be mixed again. Centrifugation should be performed in non-glass tubes (e.g., polypropylene). A centrifugal force of $20,000 \times g$ corresponds to 12,000 rpm in a Beckman JA-17 rotor or 13,000 rpm in a Sorvall SS-34 rotor. After centrifugation the supernatant should be clear.

Note: Instead of centrifugation steps 7 and 8, the lysate can be efficiently cleared by filtration using a QIAfilter Midi or Maxi Cartridge.

8. Centrifuge the supernatant again at $\approx 20,000 \times g$ for 15 min at 4°C. Remove supernatant containing plasmid DNA promptly. This second centrifugation step should be carried out to avoid applying suspended or particulate material to the QIAGEN-tip. Suspended material (causing the sample to appear turbid) can clog the QIAGEN-tip and reduce or eliminate gravity flow. Remove a 240 μ l or 120 μ l sample from the cleared lysate supernatant and save for an analytical gel (sample 1) in order to determine whether growth and lysis conditions were optimal.

9. Equilibrate a QIAGEN-tip 100 or QIAGEN-tip 500 by applying 4 ml or 10 ml

Buffer QBT, and allow the column to empty by gravity flow. Flow of buffer will begin automatically by reduction in surface tension due to the presence of detergent in the equilibration buffer. Allow the QIAGEN-tip to drain completely. QIAGEN-tips can be left unattended, since the flow of buffer will stop when the meniscus reaches the upper frit in the column.

10. Apply the supernatant from step 8 to the QIAGEN-tip and allow it to enter the resin by gravity flow. The supernatant should be loaded onto the QIAGEN-tip promptly. If it is left too long and becomes cloudy due to further precipitation of protein, it must be centrifuged again or filtered before loading to prevent clogging of the QIAGEN-tip. Remove a 240 μ l or 120 μ l sample from the flow-through and save for an analytical gel (sample 2) in order to determine the efficiency of DNA binding to the QIAGEN Resin.

11. Wash the QIAGEN-tip with 2 x 10 ml or 2 x 30 ml Buffer QC. Allow Buffer QC to move through the QIAGEN-tip by gravity flow. The first wash is sufficient to remove all contaminants in the majority of plasmid DNA preparations. The second wash is especially necessary when large culture volumes or bacterial strains producing large amounts of carbohydrates are used. . Remove a 400 μ l or 240 μ l sample from the combined wash fractions and save for an analytical gel (sample 3).

12. Elute DNA with 5 ml or 15 ml Buffer QF. Collect the eluate in a 10 ml or 30 ml tube. Use of polycarbonate centrifuge tubes is not recommended as polycarbonate is not resistant to the alcohol used in subsequent steps. Remove a 100 μ l or 60 μ l sample of the eluate and save for an analytical gel (sample 4). If you wish to stop the protocol and continue later, store the eluate at 4°C. Storage periods longer than overnight are not recommended.

13. Precipitate DNA by adding 3.5 ml or 10.5 ml (0.7 volumes) room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at $\approx 15,000 \times g$ for 30 min at 4°C. Carefully decant the supernatant. All solutions should be at room temperature in order to minimize salt precipitation, although centrifugation is carried out at 4°C to prevent overheating of the sample. A centrifugal force of 15,000 $\times g$ corresponds to 9500 rpm in a Beckman JS-13 rotor and 11,000 rpm in a Sorvall SS-34

rotor. Alternatively, disposable conical bottom centrifuge tubes can be used for centrifugation at 5000 x g for 60 min at 4°C. Isopropanol pellets have a glassy appearance and may be more difficult to see than the fluffy, salt-containing pellets that result from ethanol precipitation. Marking the outside of the tube before centrifugation allows the pellet to be more easily located. Isopropanol pellets are also more loosely attached to the side of the tube, and care should be taken when removing the supernatant.

14. Wash DNA pellet with 2 ml or 5 ml of room-temperature 70% ethanol, and centrifuge at =15,000 x g for 10 min. Carefully decant the supernatant without disturbing the pellet. Alternatively, disposable conical-bottom centrifuge tubes can be used for centrifugation at 5000 x g for 60 min at 4°C. The 70% ethanol removes precipitated salt and replaces isopropanol with the more volatile ethanol, making the DNA easier to redissolve.

15. Air-dry the pellet for 5–10 min, and redissolve the DNA in a suitable volume of buffer (e.g., TE buffer, pH 8.0, or 10 mM Tris·Cl, pH 8.5). Redissolve the DNA pellet by rinsing the walls to recover all the DNA, especially if glass tubes have been used. Pipetting the DNA up and down to promote resuspension may cause shearing and should be avoided. Overdrying the pellet will make the DNA difficult to redissolve. DNA dissolves best under slightly alkaline conditions; it does not easily dissolve in acidic buffers.

Determination of yield

To determine the yield, DNA concentration should be determined by both UV spectrophotometry and quantitative analysis on an agarose gel.

Agarose gel analysis

We recommend removing and saving aliquots during the purification procedure (samples 1–4). If the plasmid DNA is of low yield or quality, the samples can be analyzed by agarose gel electrophoresis to determine at what stage of the purification procedure the problem occurred.

QIAGEN Plasmid Mega/Giga

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This protocol is designed for preparation of up to 2.5 mg of high- or low-copy plasmid or cosmid DNA using the QIAGEN Plasmid Mega Kit, or up to 10 mg using the QIAGEN Plasmid Giga Kit. QIAGEN Plasmid

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Things to do before starting

Add the provided RNase A solution to Buffer P1 before use. Use one vial of RNase A (spin down briefly before use) per bottle of Buffer P1, to give a final concentration of 100 µg/ml.

Check Buffer P2 for SDS precipitation due to low storage temperatures.

If necessary, dissolve the SDS by warming to 37°C.

Pre-chill Buffer P3 to 4°C.

Procedure

1. Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 5–10 ml LB medium containing the appropriate selective antibiotic. Incubate for ~8 h at 37°C with vigorous shaking (~300 rpm). Use a tube or flask with a volume of at least 4 times the volume of the culture.
2. Dilute the starter culture 1/500 to 1/1000 into selective LB medium. For high-copy plasmids inoculate 500 ml or 2.5 liters medium. For low-copy plasmids, inoculate 2.5 liters or 5 liters medium. Grow at 37°C for 12–16 h with vigorous shaking (~300 rpm). Use a flask or vessel with a volume of at least 4 times the volume of the culture. The culture should reach a cell density of approximately $3\text{--}4 \times 10^9$ cells per ml, which typically corresponds to a pellet wet weight of approximately 3 g/liter medium.
3. Harvest the bacterial cells by centrifugation at $6000 \times g$ for 15 min at 4°C. $6000 \times g$ corresponds to 6000 rpm in Sorvall GSA or GS3 or Beckman JA-10 rotors. Remove all traces of supernatant by inverting the open centrifuge tube until all medium has been drained.

Note: For Giga preparations of low-copy plasmids using 5 liters of culture, volumes of Buffers P1, P2, and P3 in steps 4–6 should be doubled, due to the very large number of cells harvested. For routine Giga preparation of low-copy plasmids, additional Buffers P1, P2, and P3 may need to be purchased (see page 83) or prepared. If you wish to stop the protocol and continue later, freeze the cell pellets at –20°C.

4. Resuspend the bacterial pellet in 50 ml or 125 ml of Buffer P1.

For efficient lysis it is important to use a vessel that is large enough to allow complete mixing of the lysis buffers. We recommend a 500 ml bottle for Mega preparations and a 1000 ml bottle for Giga preparations. Ensure that the RNase A has been added to Buffer P1. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

5. Add 50 ml or 125 ml of Buffer P2, mix gently but thoroughly by inverting 4–6 times, and incubate at room temperature for 5 min. Do not vortex, as this will result in shearing of genomic DNA. The lysate should appear viscous. Do not allow the lysis reaction to proceed for more than 5 min. After use, the bottle containing Buffer P2 should be closed immediately to avoid acidification of Buffer P2 from CO₂ in the air.
6. Add 50 ml or 125 ml of chilled Buffer P3, mix immediately but gently by inverting 4–6 times, and incubate on ice for 30 min. Precipitation is enhanced by using chilled Buffer P3 and incubating on ice. After addition of Buffer P3, a fluffy white material forms and the lysate becomes less viscous. The precipitated material contains genomic DNA, proteins, cell debris, and SDS. The lysate should be mixed thoroughly to avoid localized potassium dodecyl sulfate precipitation.
7. Centrifuge at $\approx 20,000 \times g$ for 30 min at 4°C. Remove supernatant containing plasmid DNA promptly. Before loading the centrifuge, the sample should be mixed again. Centrifugation should be performed in 250 ml or 500 ml non-glass tubes (e.g., polypropylene). A centrifugal force of $20,000 \times g$ corresponds to 11,500 rpm in a Beckman JA-14 rotor or 11,000 rpm in a Sorvall GSA rotor. After centrifugation the supernatant should be clear.

Note: Instead of centrifugation steps 7 and 8, the lysate can be efficiently cleared by filtration using a QIAfilter Mega-Giga Cartridge.

8. Centrifuge the supernatant again at $\approx 20,000 \times g$ for 15 min at 4°C. Remove supernatant containing plasmid DNA promptly. This step should be carried out to avoid applying suspended or particulate material to the QIAGEN-tip. Suspended material (causing the sample to appear turbid) can clog the QIAGEN-tip and reduce or eliminate gravity flow. Remove a 120 μ l or 75 μ l sample from the cleared lysate

supernatant and save for an analytical gel (sample 1) in order to determine whether growth and lysis conditions were optimal.

9. Equilibrate a QIAGEN-tip 2500 or QIAGEN-tip 10000 by applying 35 ml or 75 ml Buffer QBT, and allow the column to empty by gravity flow. Flow of buffer will begin automatically by reduction in surface tension due to the presence of detergent in the equilibration buffer. Allow the QIAGEN-tip to drain completely. QIAGEN-tips can be left unattended, since the flow of buffer will stop when the meniscus reaches the upper frit in the column.

10. Apply the supernatant from step 8 to the QIAGEN-tip and allow it to enter the resin by gravity flow. The supernatant should be loaded onto the QIAGEN-tip promptly. If it is left too long and becomes cloudy due to further precipitation of protein, it must be centrifuged again or filtered before loading to prevent clogging of the QIAGEN-tip. Remove a 120 μ l or 75 μ l sample from the flow-through and save for an analytical gel (sample 2) in order to determine efficiency of DNA binding to the QIAGEN Resin.

11. Wash the QIAGEN-tip with a total of 200 ml or a total of 600 ml Buffer QC.

Allow Buffer QC to move through the QIAGEN-tip by gravity flow. The first half of the volume of wash buffer is sufficient to remove all contaminants in the majority of plasmid DNA preparations. The second half is particularly necessary when large culture volumes or bacterial strains producing large amounts of carbohydrates are used. Remove a 160 μ l or 120 μ l sample from the combined wash fractions and save for an analytical gel (sample 3).

12. Elute DNA with 35 ml or 100 ml Buffer QF. Use of polycarbonate centrifuge tubes for collection is not recommended as polycarbonate is not resistant to the

alcohol used in subsequent steps. Remove a 22 μ l or 20 μ l sample of the eluate and save for an analytical gel (sample 4).

If you wish to stop the protocol and continue later, store the eluate at 4°C. Storage periods longer than overnight are not recommended.

13. Precipitate DNA by adding 24.5 ml or 70 ml (0.7 volumes) room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at $\approx 15,000 \times g$ for 30 min at 4°C. Carefully decant the supernatant. All solutions should be at room temperature in order to minimize salt precipitation, although centrifugation is carried out at 4°C to prevent overheating of the sample. A centrifugal force of $15,000 \times g$ corresponds to 9500 rpm in a Beckman JS-13 rotor and 11,000 rpm in a Sorvall SS-34 rotor. Alternatively, disposable conical bottom centrifuge tubes can be used for centrifugation at $5000 \times g$ for 60 min at 4°C. Isopropanol pellets have a glassy appearance and may be more difficult to see than the fluffy, salt-containing pellets that result from ethanol precipitation. Marking the outside of the tube before centrifugation allows the pellet to be more easily located. Isopropanol pellets are also more loosely attached to the side of the tube, and care should be taken when removing the supernatant.

14. Wash DNA pellet with 7 ml or 10 ml of room-temperature 70% ethanol, and centrifuge at $\approx 15,000 \times g$ for 10 min. Carefully decant the supernatant without disturbing the pellet. Alternatively, disposable conical-bottom centrifuge tubes can be used for centrifugation at $5000 \times g$ for 60 min at 4°C. The 70% ethanol removes precipitated salt and replaces isopropanol with the more volatile ethanol, making the DNA easier to redissolve.

15. Air-dry the pellet for 10–20 min, and redissolve the DNA in a suitable volume of

buffer (e.g., TE buffer, pH 8.0, or 10 mM Tris·Cl, pH 8.5). Redissolve the DNA pellet by rinsing the walls to recover all the DNA, especially if glass tubes have been used. Pipetting the DNA up and down to promote resuspension may cause shearing and should be avoided. Overdrying the pellet will make the DNA difficult to redissolve. DNA dissolves best under slightly alkaline conditions; it does not easily dissolve in acidic buffers.

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